CasExpress reveals widespread and diverse patterns of cell survival of caspase-3 activation during development in vivo

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Abstract

Caspase-3 carries out the executioner phase of apoptosis, however under special circumstances, cells can survive its activity. To document systematically where and when cells survive caspase-3 activation in vivo, we designed a system, CasExpress, which drives fluorescent protein expression, transiently or permanently, in cells that survive caspase-3 activation in Drosophila. We discovered widespread survival of caspase-3 activity. Distinct spatial and temporal patterns emerged in different tissues. Some cells activated caspase-3 during their normal development in every cell and in every animal without evidence of apoptosis. In other tissues, such as the brain, expression was sporadic both temporally and spatially and overlapped with periods of apoptosis. In adults, reporter expression was evident in a large fraction of cells in most tissues of every animal; however the precise patterns varied. Inhibition of caspase activity in wing discs reduced wing size demonstrating functional significance. The implications of these patterns are discussed.

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Introduction

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I. Apoptosis: an overview

Apoptosis is a process of programed cell death that is characterized by its characteristic morphological changes as well as its biochemical pathways. The term apoptosis is derived from the ancient Greek word ἀπόπτωσις, which means “falling off”. This term was initially introduced by JF Kerr’s epochal literature in 1972, which describes an inherently programmed phenomenon that controls cell deletion in mammals during normal development or pathological conditions(Kerr, Wyllie, & Currie, 1972). In Kerr’s research，apoptosis initially was identified and described by its morphological features such as condensation, fragmentation and consumption by phagocytosis. However the molecular mechanism of apoptosis remained unknown for many years until it was revealed by Horvitz’s work on programmed cell death during the development of *Caenorhabditis* elegan(Ellis & Horvitz, 1986).

Apoptosis is considered to be important to maintain the homeostasis and normal development of multicellular organisms. Besides its homeostatic function of shaping and maintaining the normal tissues *in vivo*, apoptosis also eliminates detrimental or damaged cells through defensive processes like immune responses (Everett &

McFadden, 1999). In a living organism, apoptosis can be triggered by various stimuli and conditions including toxins, radiation, chemotherapy drugs or DNA damage. In certain types of cells, it can also be triggered by the acquisition of death signals or the deprivation of survival signals. It is the kind of stimulations and the dosage of stimuli that determine through which mechanism a cell dies (Elmore, 2007).

It is to be noted that there are other alternative types of cell death with completely different mechanisms and morphological features such as necrosis or autophagy. In apoptosis, the dying cells split and form apoptotic bodies which are sealed by cell membrane, and cell's content would not be release to the environment. Where as in necrosis, cell membrane ruptures and the cell's content gets released. In apoptosis, the apoptotic bodies will sequentially be consumed by surrounding cells. While in the autophagy, double-membrane autophagosomes are formed inside cells. The autophagosomes then fuse with an organelle called lysosome that is filled with various hydrolytic enzymes. The contents within autophagosomes will be digested.

Apoptosis is a process that presents universally in different multicellular organisms, from a relatively simple nematode *C. Elegans,*

which is composed of around a thousand somatic cells, to an extremely complicated mammal like *Homo Sapiens*, which is composed of hundreds of trillions of cells. Many essential morphological and biochemical characteristics of apoptosis are highly conserved throughout the process of evolution, including the activation of a group of cysteine-aspartic proteases called “caspases” and the cascade of proteolytic events following caspase activation.

In humans, inappropriate apoptosis can cause dire health problems. Excessive apoptosis results in various diseases including ischemic damage(Gottlieb, Burleson, Kloner, Babior, & Engler, 1994), and neurodegenerative diseases(Friedlander, 2003), while absence from necessary apoptosis leads to autoimmune diseases(Eguchi, 2001) or cancer(Kerr, Winterford, & Harmon, 1994). Thus the mechanism of apoptosis is exceedingly important to the development of diagnostic and therapeutic approaches to these diseases.

II. Initiation of apoptosis and activation of caspase

Conditions including binding of death inducing factors, deprivation of growth factors, nutrients, or damage of a cell induced by radiation, toxins, injuries, chemotherapy drugs or other perturbations can initiate apoptosis. Current studies showed that two pathways generally induce apoptosis: the intrinsic pathway through mitochondria or the extrinsic pathway through receptors on cell membrane(Fulda & Debatin, 2006). Research have shown that these two pathways in some situations are connected and do interfere with each other (Igney & Krammer, 2002; Li, Zhu, Xu, & Yuan, 1998). In both pathways, upstream cascades lead to the activation of pro-enzymes of so-called "initiator" caspases, such as caspase-8, 9 or 10. The activated initiator caspases consequentially cleave the pro-enzymes of downstream "executioner" caspases including caspases-3. Executioner caspases cleave numerous downstream substrates in the cytoplasm and nucleus and result in morphological changes including blebbing, cell shrinkage, nuclear condensation and DNA fragmentation.

Extrinsic pathway

In the extrinsic pathway (Fig. 1), apoptosis activation is mediated by trans-membrane death receptors that belong to the tumor necrosis factor receptor protein superfamily. Each of these cytokine receptors has 2-4 extracellular cysteine-rich domains that grant it the ability to bind Tumor Necrosis Factors (TNFs). TNF receptors play roles in apoptosis, inflammation, survival, proliferation and differentiation. Among them a sub-group called death receptors include TNFR1, Fas receptor, DR4 and DR5, all of which contain a death domain (DD), which is a protein motif about 80 amino acid residues in length that contains a bundle of six alpha helices. The death domain is essential for the receptor to transmit the extracellular ligand signal to intracellular signaling cascades. Generally there are two models of the extrinsic pathway in human: the indirect

TNF-alpha model and direct Fas model (Pradeep Nair, 2014).

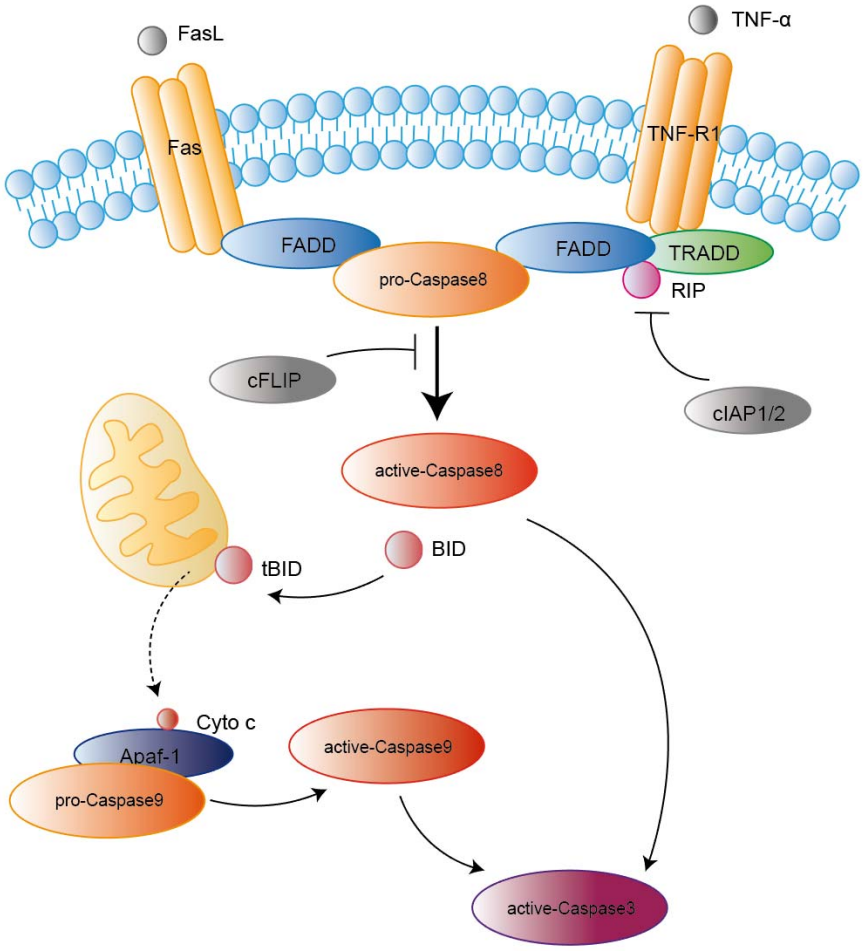

Figure 1. Extrinsic Pathway of Apoptosis

In the Fas model, binding of ligands such as FasL to its receptor Fas (also known as CD95, APO-1) or TRAIL to its receptors DR4 and DR5 results in the formation of a death-inducing signaling complex (DISC) (Pan et al., 1997; Waring & Mullbacher, 1999). Upon ligand binding, the

receptors' death domain (DD) binds to the death domain of an adapter molecule FADD. This binding exposes FADD's death effector domain (DED), which subsequently recruits the initiator caspase-8, forms the DISC and result in caspase-8's proteolytic self-cleavage. The self-cleavage of caspase-8 produces two subunits, p10 and p18, and two of each of these subunits assemble to form a heterotetramer that is an active initiator caspase (Lavrik et al., 2003).

In the TNF-alpha model, on the other hand, the TNF-alpha ligand binds to TNFR1 and results in recruitment of an adapter protein, TRADD. The consequent recruitment of FADD and caspase-8 by TRADD results in the activation of caspase-8 in a manner similar to the Fas model. After the activation of caspase-8, in some circumstances caspase-8 activates executioner caspases directly. While in other cases, caspase-8 cleaves the Bcl-2 family protein BID and activates the mitochondrial apoptosis pathway (Kantari & Walczak, 2011; Li et al., 1998). In either case, the activation of caspase-8 triggers the execution phase of apoptosis.

The extrinsic pathway is also regulated by inhibitor of apoptosis (IAP) proteins as well as other regulators. In TNF Receptor-I mediated apoptosis, the kinase RIP1 is crucial for the formation of the cytoplasmic

complex that activates caspase-8 (Micheau & Tschopp, 2003). The IAP proteins c-IAP1 and c-IAP2 can prevent the formation of the complex by catalyzing the ubiquitination of RIP1 because they are E3 ubiquitin ligases. In addition to the IAPs, c-FLIP is another regulator of extrinsic pathway. C-FLIP (cellular caspase-8 (aka FLICE) like inhibitory protein) inhibits apoptotic signals by competing FADD with caspase-8. It is regarded as a key regulator of extrinsic pathway that suppress signaling from multiple death receptors including Fas and TNF Receptor-I (Bagnoli, Canevari, & Mezzanzanica, 2010).

Intrinsic pathway

In addition to the extrinsic pathway, apoptosis can also be activated through the intrinsic pathway (Fig. 2) in vertebrates. The intrinsic pathway of apoptosis can be triggered by stimuli including radiation, toxins, chemotherapy drugs, free radicals, microbe infection or hypoxia. In addition, it can be activated by deprivation of pro-survival factors such as nutrients, survival signals, or hormones. These stimuli transduce signal through membrane receptors, in the cases of growth factors, or through cytosolic sensors, in the cases of UV or DNA (Elmore, 2007).

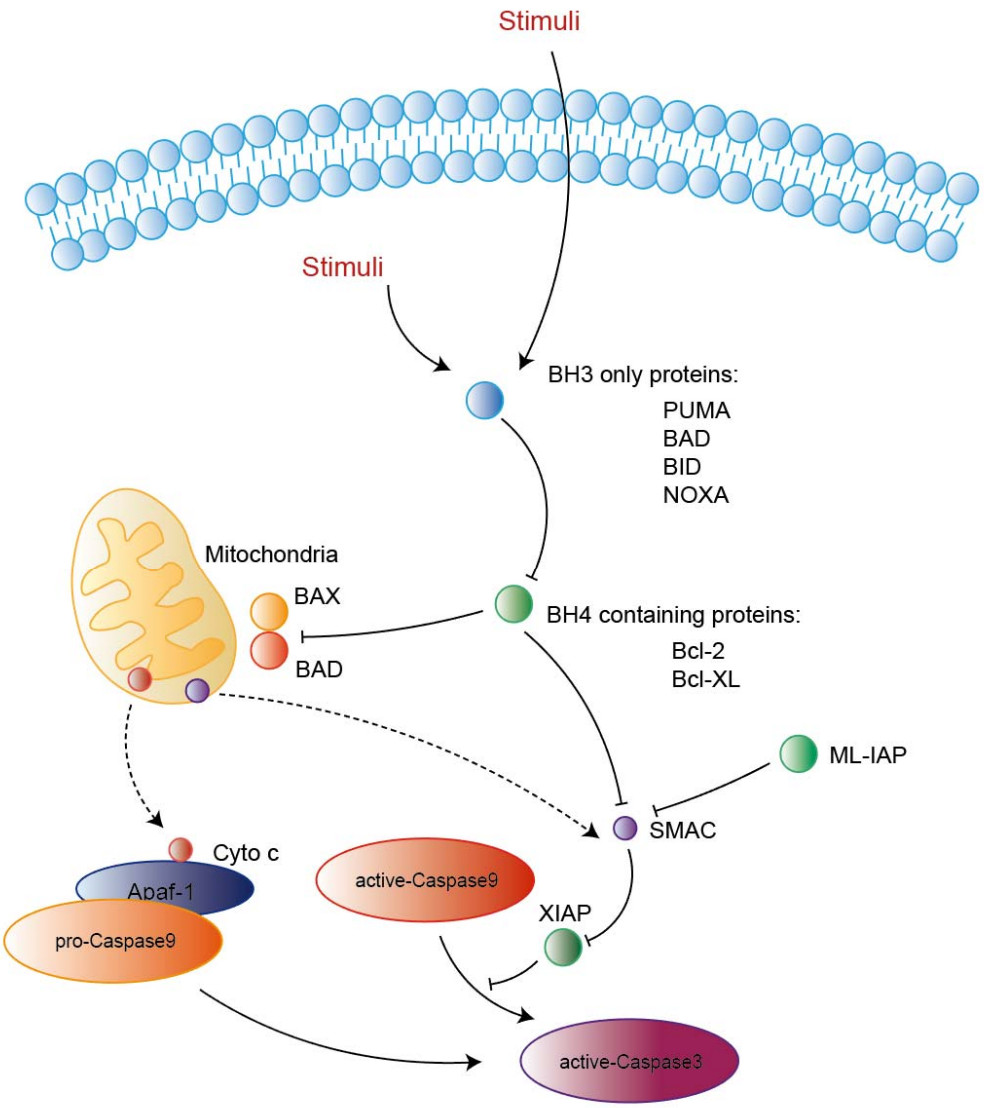

Figure 2. Intrinsic Pathway of Apoptosis

damage. Many of these stimuli converge on the activation of the tumor suppressor p53(Mercer, Mahmoudi, & Bennett, 2007), a key regulator of apoptosis and DNA repair. One of the most important mechanisms

through which these pathways regulate apoptosis is the regulation of "BH3 only" pro-apoptotic proteins in multiple levels.

Cytochrome c is the key component to the activation of the mitochondrial pathway of apoptosis. Cytochrome c is an essential part of the electron transport chain (ETC), which transfers electrons from electron donors to electron receptors though red-ox reactions and generates a proton gradient, which is essential for ATP synthase to drive the synthesis of ATP. Unlike other components of the ETC, cytochrome c is water soluble and associates with the inner membrane of mitochondria loosely through its binding to inner membrane lipid Cardiolipin. Upon the activation of mitochondrial apoptotic pathway, the mitochondria become permeable and as a consequence the cytochrome c is released to cytosol(Yang et al., 1997). The cytosolic cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) upon its release, causing the formation of a wheel-like structure of Apaf-1 heptamer called the apoptosome(Cecconi, Alvarez-Bolado, Meyer, Roth, & Gruss, 1998). The activated Apaf-1 proteins on the apoptosome will then recruit caspase-9, another initiator caspase similar to caspase-8 in the extrinsic pathway, and activate it. The activation of caspase-9 will then lead to the activation

of executioner caspases and the downstream consequences of apoptosis(Yuan & Akey, 2013).

The intrinsic pathway of apoptosis is tightly regulated at multiple steps to make sure that cells do not kill themselves by accident or in improper scenarios. Some of these regulatory events happen before the release of cytochrome c, essentially by Bcl-2 family proteins, the key regulators of mitochondrial permeability in apoptosis. In addition, there are regulatory events after the release of cytochrome c, by inhibitors of apoptosis (IAPs) including ML-IAP and XIAP, and their neutralizers that are called anti-IAP proteins.

Bcl-2 family proteins are mitochondrial membrane proteins that regulate the release of pro-apoptotic proteins, especially cytochrome c. There are two types of Bcl-2 family proteins: pro-apoptotic Bcl-2 and anti-apoptotic Bcl-2. Pro-apoptotic Bcl-2 promotes the apoptotic reactions by enhancing the permeability of mitochondria, while anti-apoptotic Bcl-2 proteins acts in an opposite way by inhibiting the permeability. In addition, these two groups of proteins interact with each other by forming heterodimers and inhibit each other as a consequence.

The Bcl-2 protein itself, as an anti-apoptotic protein, contains four Bcl-2 homologous regions: BH1, BH2, BH3, BH4. All other anti-apoptotic Bcl-2 family proteins, including Bcl-$X_{L,}$ Bcl-x, Bcl-X_S, Bcl-w and BAG, contain homologs of all these four regions. While there are two categories of pro-apoptotic Bcl-2 proteins: Bcl-2 effector proteins and BH3 only proteins. Bcl-2 effectors proteins, for example BAX and BAK, have BH1-3 regions but lack the BH4 region. The BH3 only proteins, including BID and BAD, only contain BH3 regions(Czabotar, Lessene, Strasser, & Adams, 2014).

In a healthy cell, the pro-apoptotic protein BAK is anchored on the outer membrane of mitochondria through its trans-membrane domain (Griffiths et al., 1999), whereas BAX mainly presents in the cytosol with its trans-membrane domain binds to its own hydrophobic groove. Upon the activation of mitochondrial apoptotic pathways, upstream signals promote the conformational changes of the BAX, which expose its trans-membrane domain and anchor the proteins to mitochondrial outer membrane. BAK and BAX exist mainly as inactive monomers in normal conditions. During apoptosis, however, these two Bcl-2 effector proteins undergoes conformational charges and form oligomers that

permeablizes mitochondria outer membranes and allow pro-apoptotic proteins such as cytochrome c, Smac/Diablo or HtrA2/Omi to be released to cytosol. The formation of Bcl-2 effector oligomers may be facilitated by BH3 only proteins like BID or BIM(Czabotar et al., 2014).

The anti-apoptotic Bcl-2 proteins including Bcl-2 and Bcl-X_L are located on the outer membrane of mitochondria. They bind to Bcl-2 effector proteins and prevent them from forming the oligomers that release cytochrome c and other pro-apoptotic intermembrane proteins to cytosol. It is reported that in healthy cells BAX regularly translocates to mitochondria membrane without induction. Anti-apoptotic protein Bcl-X_L retrotranslocates BAX to the cytosol to prevent unsolicited apoptosis (Edlich et al., 2011).

Most of the Bcl-2 family members are of the BH3 only protein subfamily, of which the major members include BIK, BIM, BMG, BID, BAD, BNIP3, NOXA, PUMA and EG1-1. One important function of BH3 only proteins is inhibiting anti-apoptotic Bcl-2 proteins through protein-protein interactions between alpha-helix containing BH3 domains of both sides. For example, BH3 only proteins BAD (Petros et al., 2000) or BIM (Liu, Dai, Zhu, Marrack, & Kappler, 2003) interact with Bcl-X_L

through BH3 domains. Such interactions neutralize anti-apoptotic proteins' ability to inhibit pro-apoptotic Bcl-2 effectors, thus facilitate the release of intermembrane pro-apoptotic proteins. BH3 only protein in some case may also interact with BAK or BAX or facilitate the formation of their oligomers.

BH3 only proteins play important roles as connections between stimuli and the downstream pathways of apoptosis. For example, the accumulation of tumor suppressor p53, in response to severe DNA damages or stress, induces apoptosis through upregulation of BH3 only protein PUMA (p53 upregulated modulator of apoptosis) (Nakano & Vousden, 2001) or Noxa (Oda et al., 2000). Furthermore, many pro-survival signals inhibit apoptosis by downregulating the activity (Datta et al., 2000) or amount (Ley, Balmanno, Hadfield, Weston, & Cook, 2003) of BH3 only proteins. The activity of BH3 only proteins are regulated on the multiple levels, including transcription, RNA stability, protein stability, post-translational modifications, protein conformation and subcellular localization (Lomonosova, Ryerse, & Chinnadurai, 2009). It is worth mentioning that the cleavage and activation of BH3 only protein

BID by caspase-8 links the extrinsic pathway of apoptosis to the intrinsic pathway.

The extrinsic pathway of apoptosis is also regulated by IAP (Inhibitor of Apoptosis Protein) family proteins. XIAP (X-linked inhibitor of apoptosis protein) belongs to (IAP) family and acts as an important regulator of the intrinsic pathway. XIAP utilizes its BIR2 domain to bind to executioner caspases like caspase-3 or caspase-7, or its BIR3 domain to bind to initiator caspase-9, and inhibit these caspases through bindings (de Almagro & Vucic, 2012). XIAP also contains a RING domain on its C-terminus that can inhibit apoptosis through its ubiquitin ligase activity.

Upon the activation of the mitochondrial apoptotic pathway, pro-apoptotic intermembrane mitochondrial proteins Smac/Diablo and HtrA2/Omi are release to the cytosol alongside with cytochrome c. Smac binds to IXAP through its IAP-binding motif and inhibits its binding to caspases. Similar to Smac, HtrA2 binds to IXAP and inhibits it. In addition, HtrA2 inactivates IXAP by proteolytically cleaving it(Vaux & Silke, 2003).

Execution phase of apoptosis and morphological consequences

As is demonstrated above, different signaling pathways, whether they are extrinsic or intrinsic, lead to the activation of apoptosis by converging on the activation of executioner caspases. There are three major executioner caspases in mammal, caspase-3, caspase-6 and caspase-7(Elmore, 2007). These caspases share common targets, and each has unique cleavage targets. In total, executioner caspases cleave at least hundreds of targets during the process of apoptosis, and these cleavage events are responsible for the demolition of the cell.

Contrary to another cell death process, “necrosis”, in which the cell membrane breaks and the cellular contents are release, apoptosis breaks the cell into several apoptotic bodies that are sealed by cell membrane. This is the consequence of a series of morphological changes including cell contraction, membrane blebbings, chromatin condensation, nuclear fragmentation and removal from the epithelia for epithelial cells. The rearrangement of cytoskeleton mediated by caspase cleaved components is essential for these morphological changes(Ndozangue-Touriguine, Hamelin, & Breard, 2008).

Executioner caspases activate various signaling cascades by proteolytic cleavages to weaken the cell structure and tear the cell apart.

ROCK1 is one of the most important components that executioner caspase cleaves for the modification of cell skeleton. ROCK1 is a widely expressed kinase that acts as a regulator of cell dynamics. ROCK1 is usually activated by Rho family GTPases, including Rac, Rho and Cdc42. Caspase-3 but not caspase-6 or caspase-7 generates a constitutively activated ROCK1 independent of Rho activity by exposure of its kinase domain through cleavage. Caspase mediated cleavages also cleave PAK2 and activate it in a similar manner as ROCK1 is activated. ROCK1 and PAK2 phosphorylate myosin light chain (MLC) and therefore increase the contractility of actin-myosin system and cause the membrane blebbings (Ndozangue-Touriguine et al., 2008). Since the actin filaments attach to the nuclear envelopment, the actin-myosin contraction mediated by caspase also results in nuclear fragmentation by tearing the nuclear apart (Croft et al., 2005).

In addition, executioner caspases also regulate the cytoskeleton by cleaving its components directly. For example, caspase-3 cleaves nuclear lamina, which result in chromatin condensation. In addition, executioner caspases also cleave other intermediate filaments like keratins and vimentin, and demolish their cytoplasmic networks as

consequence(Morishima, 1999). Other cytoskeletal components, including actin and some of the f-actin binding proteins, tubulin and several microtubule-associated proteins are also targeted by executioner caspases (Ndozangue-Touriguine et al., 2008; Taylor et al., 2007).

Apoptosis is also able to cause DNA damage and nuclear fragmentation in mammals. Upon mitochondrial outer membrane permeabilization, pro-apoptotic proteins AIF and EndoG are released from the mitochondria to cytosol and cause early stage of the DNA condensation (Susin et al., 2000). Executioner caspases cleave the inhibitor of CAD (ICAD) in the later stage of apoptosis, and result in its dissociation with CAD (caspase-Activated DNase). The released CAD then forms oligomers and facilitate the late stage of DNA condensation with its DNase activity (Susin et al., 2000). In *Caenorhabditis elegans*, however, DNA damage does not occur until the apoptotic cell is consumed by cell engulfment (Wu, Stanfield, & Horvitz, 2000). There are several hypotheses about the purpose of DNA damage. One of hypotheses is that this destruction occurs to avoid autoimmune disease. DNA is frequently recognized as a self-antigen in autoimmune disease such as systemic lupus erythematosus. Previous research has suggested that

mouse embryos with deficiency of DNA degradation go on to produce adults with abnormality of their immune system and thymic development (Kawane et al., 2003). In addition, degradation of DNA might be a strategy to destroy the genomes of invading viruses (Taylor, Cullen, & Martin, 2008).

Apoptosis demolishes the housekeeping functions of a cell in multiple ways. The mitochondrial outer membrane permeabilization induced by Bcl-2 family proteins results in the fragmentation of mitochondria. Executioner caspases also cleave other housekeeping genes and shut down the normal functions of a cell. For example, caspases shut down protein translation by cleavage of multiple translation initiation factors as well as ribosomal subunits (Luthi & Martin, 2007). Caspase mediated proteolytic cleavage events also lead to the fragmentation of the Golgi (Lane, Vergnolle, Woodman, & Allan, 2001) and ER (Lane, Allan, & Woodman, 2005).

III. Anastasis: A reversal of apoptosis

After induction, apoptosis is generally a rapid and dramatic process. It is common that the activation of caspase and the process of apoptosis happen within minutes after mitochondrial outer membrane

permeabilization (MOMP) (Chipuk, Moldoveanu, Llambi, Parsons, & Green, 2010). As is described in the passages above, the MOMP and activation of caspase is sufficient to result in cell death, as it leads to the undermining of cell structure as well as the damage of cellular housekeeping functions such as aerobic respiration, protein translation and protein maturation.

While caspase activation is frequently a terminal event resulting in swift cellular demise (Chang, Putcha, Deshmukh, & Johnson, 2002), cell survival following caspase activation has been described (e.g., [(Florentin & Arama, 2012); (Kuranaga & Miura, 2007); (Kumar, 2004); (Meinander et al., 2012)]).

Cells can survive caspase activation following a lethal dose of an apoptotic stimulus, as long as it is transient and thus sublethal in time. Such reversal can happen after different hallmarks of apoptosis, including the activation of caspase-3, the cleavage of its downstream targets like PARP, as well as the morphological consequences of caspase-3 activation like blebbing or nuclear condensation (Tang et al., 2012). This reversal of late stage apoptosis has been named anastasis (ἀνάστασις, Greek for 'rising to life').

Anastasis has been observed in a variety of cell types including cancer cell lines, NIH 3T3 fibroblasts, as well as primary cultures such as mouse primary liver cells and rat primary heart cells. It has also been observed following induction of apoptosis with different stimuli, for example ethanol or DMSO (Tang et al., 2012). Anastasis has also been observed following apoptosis induction by classic inducers, such as staurosporine.

Anastasis can occur even after apoptosis-induced DNA damage. While most cells fully recover and repair their damaged DNA, a small fraction of cells that have undergone anastasis survive with mutations or chromosomal abnormalities. A tiny fraction of NIH 3T3 cells that undergo anastasis (20 out of 10^7) exhibit signs of oncogenic transformation such as focus formation and proliferation in soft agar. These phenotypes are not detected in untreated controls. These results are presumably the consequence of imperfect DNA repair during the process of anastasis (Tang et al., 2012). The oncogenic transformation following anastasis could be a potential explanation of higher liver cancer morbidity among alcoholics as well as pathogenesis of other tumors (McKillop & Schrum, 2005; Tang et al., 2012). Anastasis also could in principle allow tumor cells to resist and escape chemotherapy (Tang, Yuen, Tang, & Fung, 2009).

Anastasis has been observed in many different cell types, in response to different stimuli, and no artificial genetic alteration is required to induce it. This strongly implies that cells possess an intrinsic mechanism to allow recovery from the brink of apoptosis, and that such a mechanism likely has physiological purposes. Although anastasis has the potential to lead to genomic alterations and oncogenic transformation, it is not likely that organisms evolved this capability to allow cancer to develop or to escape chemotherapy. We propose that organisms have evolved anastasis for some beneficial purpose. Anastasis may be a mechanism for organisms to protect the cells that are important and very hard to replace. For example, anastasis might limit permanent damage to the heart following transient ischemia (Kenis et al., 2010), or protect nerve cells from stroke or neuronal degenerative diseases.

Observations made in Drosophila and mammals have shown that cells can survive following caspase activation in response to a sublethal dose of irradiation. In certain circumstances such sublethal doses of caspase activation could also promote DNA damage and oncogenic transformation similar to anastasis (Florentin & Arama, 2012; Ichim et al., 2015; Liu et al., 2015). In addition, it has also been reported that in

some cells and tissues, caspases promote localized or partial destruction of the cell without actually killing it (Arama, Agapite, & Steller, 2003; Connolly, Jager, & Fearnhead, 2014; Huh et al., 2004). These observations also suggest that cells possess other mechanisms, besides anastasis, to protect themselves from the destructive effects of caspase activity. It would be interesting to understand the common ground and relationship between the mechanism of cell survival under a sublethal dose of caspase activity, and the mechanism of anastasis, which is cell survival following a transient lethal dose of caspase activity.

IV. The tool box of fly genetics

Drosophila melanogaster, also known as the common fruit fly, belongs to the family *Drosophilae*. Beginning with the work of Charles W. Woodworth, fruit flies have been used as a model organism for more than a century. Utilizing *Drosophila*, Thomas Hunt Morgan achieved his epoch-making breakthroughs in genetics, by making clear the role of chromosomes in heredity.

Since the times of Morgan, the fruit fly has been one of the most studied model organisms. Scientist developed various protocols like EMS mutagenesis (Keightley, 1996) and P-element insertion (Sundaresan et

al., 1995) to generate mutated alleles and study the functions of genes. The transposon P-element has also been used to insert transgenes into fly genomes. Scientists also developed tools like balancers and genetic markers to map the location of their favorite gene alleles. Many of these tools preceded PCR or whole-genome sequencing. These tools make *Drosophila* an extremely versatile model for genetics study. Many breakthroughs in most fields of biology, including developmental biology, neuroscience, molecular biology, cell biology, or evolutionary biology have been made in fruit flies, and many of the most intensively studied genes have been named after their original phenotypes identified in *Drosophila*, like Wnt, Hippo, or Hedgehog.

Scientists continue to develop new genetic tools in *Drosophila* to address new questions. An important goal is to control which transgenes will be expressed and where that expression happens in flies. This can be achieved by binary expression systems, among which the Gal4-UAS is most commonly used(Duffy, 2002) (Fig3. A).

Gal4 is a yeast transcription factor, and UAS is the DNA sequence it binds to facilitate the expression of the downstream sequence. Scientists have generated numerous Gal4 strains in Drosophila by either inserting

the Gal4 sequence into the locus of a gene, or inserting open reading frame of a Gal4 that is flanked by several kilo-bases of DNA sequences, which are cloned from the 5' and 3' of a target gene from fly genome. In this way, the expression pattern of Gal4 in the transgenic strain will follow the pattern of that gene.

In addition, scientists have also generated a collection of UAS strains, by putting wild-type ORF, altered variants like constitutively active or dominantly negative versions, or short-hairpin RNA of genes downstream of several tandem UAS sequences, and inserting these plasmids into the fly genome. After crossing these transgenic flies with certain Gal4 strains, these sequences could be expressed following the Gal4 strains' expression patterns(Duffy, 2002). It is worth mentioning that there are several other binary expression systems like QF-QUAS (Potter & Luo, 2011) or LexA-LexAop (Lai & Lee, 2006) that can work in parallel with UAS-Gal4. These systems can thus be used together to perform

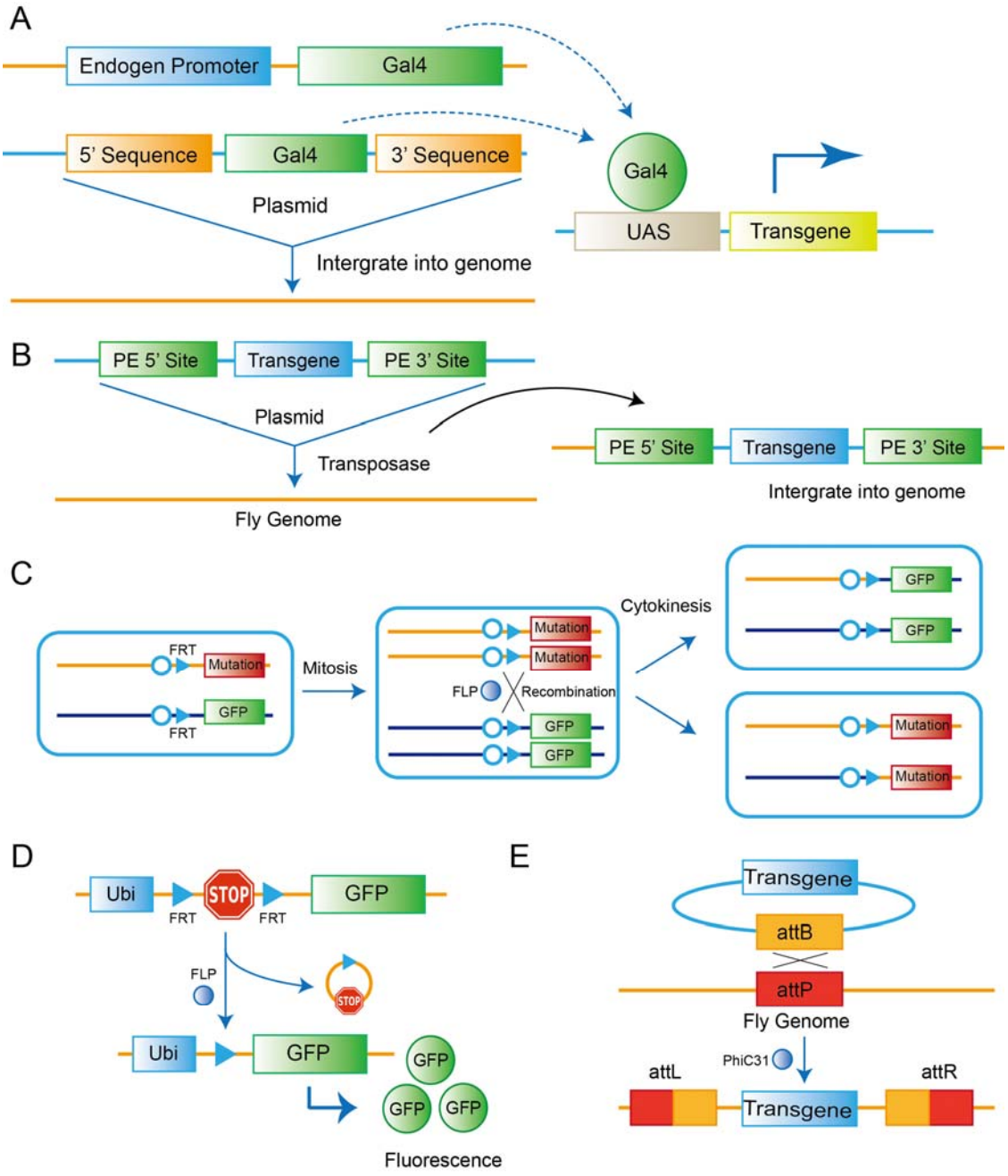

Figure 3. Scheme of several fly genetics tools
A. Gal4-UAS binary expression system B. P-element insertion of a transgene into genome C. FRT/FLP induced mosaic clone D. FRT/FLP induced linage tracing E. Site-directed insertion of transgene mediated by PhiC31 intergrase

complicated genetics experiments.

Scientists have also worked really hard to figure out versatile ways of manipulating the fly genome, like inducing recombination, or inserting or deleting certain DNA sequences. The transposon P-element mentioned above is one of the very common tools used to achieve this purpose. In the presence of P element transposase, the DNA sequence in a plasmid flanked by 5' and 3' elements of P element can by inserted into random locations of a fly genome (Robertson et al., 1988) (Fig3. B).

Utilizing P-Element insertion, researchers are able to embed recombination sites, which are DNA sequences that are recognized by recombinase enzymes, into the fly genome. Upon the presence of the corresponding recombinase enzymes, DNA recombination can be induced between these recombination sites. The most commonly used recombination site/recombinase pair in flies is yeast-derived FRT/FLP. Besides FRT/FLP, Gerald M. Rubin's group has generated several other pairs with high-fidelity like B3/B3RT(Nern, Pfeiffer, Svoboda, & Rubin, 2011). Recombination is used in flies to generate mosaic clones, which are clones of homozygous genotype of an allele within a fly of heterozygous genotype (Xu & Rubin, 1993)(Fig3. C). For alleles of which

the homozygous genotype is lethal in a whole fly, mosaic analysis allows the analysis of homozygous genotype in certain groups of cells.

Recombination can also be used for lineage tracing (Fig3. D). In lineage tracing, the expression of recombinase is usually trigged randomly. After the triggering, the expressed recombinase FRT can permanently remove a transcriptional stop sequence that is flanked by two FLP sites in genome. Consequently, the DNA sequence following this removed sequence, usually the ORF of a fluorescent protein, will be expressed in this cell and its progeny under the control of a ubiquitously expressed promoter(Evans et al., 2009).

Recombination can also be used to generate site-directed-insertion of transgenes in flies. Several labs have generated a collection of fly strains with attP sequencing inserted in fly genome by P-Element insertion. AttP is a recombination sequence of bacteriophage derived integrase PhiC31(Fig3. E). Following induction by PhiC31, a plasmid can be specifically integrated into the attP site in the fly genome(Groth, Fish, Nusse, & Calos, 2004). Compared to P Element induced way of generating transgenic flies, site-directed-insertion can avoid the disruption of other genes by inserting into or around them. Also the expression patterns of

many of these attP sites are well characterized. Some of these sites such as attP2 or attP40 (Perkins et al., 2015) express genes at mild levels that are suitable for most of the applications, and unwanted “leaky” expression is low at the same time.

Summary

Above I introduced the programmed cell death process of apoptosis as well as its reversal process. The observations of anastasis in mammalian cell culture strongly imply that anastasis has physiologically relevant functions in vivo, such as protecting cells that are very important or hard to be replaced. Also anastasis in vivo might be related to oncogenesis as well as the tumor's resistance to chemotherapy drugs. Thus identifying and characterizing cells undergoing anastasis and survive becomes a very important and interesting research aim. However, it is not easy to chase such a process in vivo because most of the molecular characteristics of anastasis remain unknown and there is no reliable molecular marker for anastasis. The powerful fly genetics tools provide a great potential to solve this problem, by connecting the event of caspase-3 activation within a cell to a series of molecular events that finally lead to the permanent modification of the cell’s genome. My thesis

research is aimed at creating such tools to identify and characterize the cells that survive caspase-3 activity in vivo.

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Chapter I

CasExpress Reveals Large-scale Survival of Caspase-3 Activity *in vivo* in *Drosophila* Melanogaster

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Abstract

Caspase-3 carries out the executioner phase of apoptosis, however under special circumstances, cells can survive its activity in the process of anastasis. To study anastasis and document systematically where and when cells survive caspase-3 activation in vivo, we designed a lineage tracing system called CasExpress in transgenic flies. Using a combination of the binary UAS-Gal4 expression system and the FLP-FRT recombination system, CasExpress is able to drive fluorescent protein expression, transiently or permanently, in cells that survive the activation of caspase-3 homologs DrIce and Dcp-1 in Drosophila. We demonstrated its specificity to capase-3 by knocking out or inhibiting caspases. Utilizing CasExpress, we discovered widespread cell survival following caspase-3 activation in adult and larvae of *Drosophila melanogaster*. We also characterized its pattern of expression in different developmental stages.

Introduction

A variety of primary cells and cell lines can survive caspase activation following an otherwise lethal dose of an apoptotic stimulus in limited time in a process called anastasis, as long as that stimulus is transient and thus sublethal (Tang et al., 2012). Such survival following caspase activation has the potential for both beneficial and harmful effects. It may limit permanent damage to the heart following transient ischemia (Kenis et al., 2010); however it might also be oncogenic (Tang et al., 2012; Liu et al., 2015; Ichim et al., 2015), and could in principle allow tumor cells to escape chemotherapy.

Apoptosis is a critical feature of normal development in multicellular organisms (Miura, 2012; Denton and Kumar, 2015; Vaux and Korsmeyer, 1999). Studies in model organisms such as worms and flies have made important contributions to unraveling the underlying mechanisms (Connolly et al., 2014; Denton and Kumar, 2015; Orme and Meier, 2009;

Steller, 1995). It is unknown whether cells ever recover from the brink of apoptotic cell death during development. The observations that cultured cells and adult cardiac myocytes can recover from transient insults that cause caspase-3 activation raised the question as to how widespread cell survival following caspase activation might be in vivo, whether this ever occurs during normal development, and if so what function it might serve.

Identification of cells that survive transient caspase activation is challenging because they bear no known distinguishing characteristic. Therefore we developed a genetic system to mark and manipulate cells that survive caspase activation in *Drosophila* (Figure 1). Using these CasExpress transgenic flies, we discovered that the majority of cells in the adult derive from cells that survive caspase activation during normal development.

Results

Design of CasExpress, an in vivo sensor for cells that survive caspase activation

In order to detect and follow the fates of cells that survive caspase activation, we designed a caspase-inducible Gal4 transcription factor (Figure 1A). To keep Gal4 inactive in the absence of caspase activity, we tethered it to the plasma membrane by fusing it to mCD8 (mouse cluster of differentiation 8). To render the protein caspase-inducible, we inserted the caspase-3-binding and cleavage domain from the Drosophila Inhibitor of Apoptosis Protein 1 (DIAP1) (Ditzel et al., 2003) in between CD8 and Gal4. As a negative control we created a second transgene with a DQVD to DQVA amino acid substitution in the caspase cleavage site (Figure 1B) in order to render it caspase insensitive, hereafter the 'DQVA control.' To allow for detection of caspase activation in as many cell types as possible, the fusion

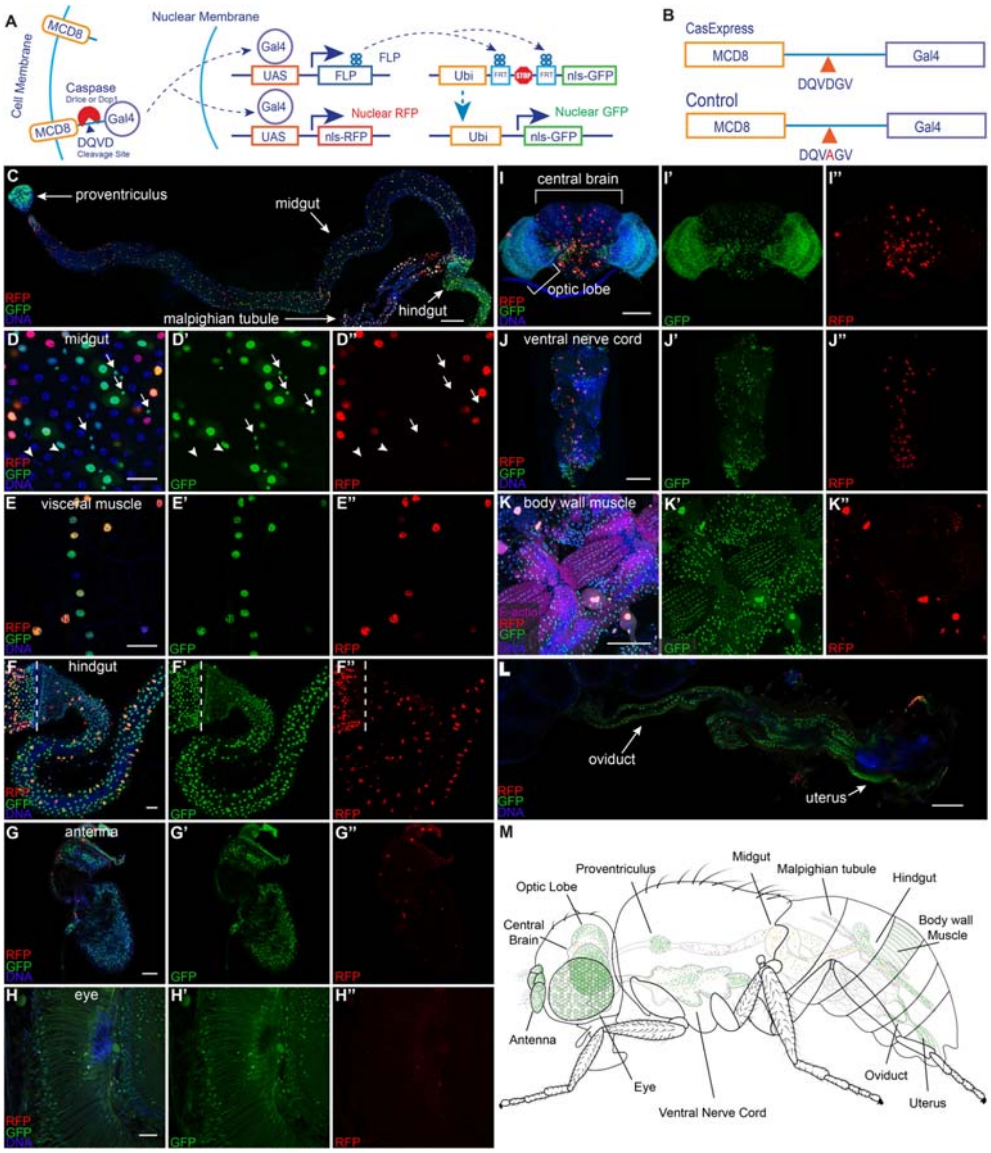

Figure 1.**Widespread CasExpress activation in adult tissues.**

(**A**) A schematic of CasExpress and G-trace. (**B**) A schematic showing the sequence of the DQVD caspase cleavage site in CasExpress and the point mutation in the DQVA control. (**C–L**) Confocal micrographs showing overlays of DAPI, RFP and GFP from CasExpress/G-Trace flies. (**D'–L'**) GFP channel only. (**D''–L''**) RFP channel only. Arrows in **D–D''** indicate examples of GFP+ progenitor cells, and arrowheads point to examples of GFP- progenitor cells. Dotted lines in **F–F''** mark the boundary between midgut and hindgut. Scale bars in C and I-L are 100 μm; scale bars in **D–H** are 25 μm. (M) A schematic summarizing the general pattern of GFP and RFP expression in adult. Although GFP expression was present in all body wall muscle, only part is shown in green for simplicity and presentation clarity.

protein was expressed under the control of the ubiquitin (ubi) enhancer/promoter. We characterized the expression and activity of transgenic flies bearing a site-directed insertion of this transgene into the attP40 landing site, selected for its ability to allow relatively uniform, moderate levels of expression in a variety of tissues (Markstein et al., 2008). We also confirmed that the DQVD sensor and DQVA control showed similar patterns and levels of cell surface fusion protein expression, detected with anti-mCD8 antibody staining, throughout the embryo and in most tissues and stages of development (Figure 2). We named this system CasExpress for its ability to drive expression of downstream genes and proteins under the control of caspase-3 activity.

Widespread activation of CasExpress in the adult

To detect caspase activity, we crossed the sensor and control to G-Trace (Evans et al., 2009) a fly line that expresses two fluorescent protein targets, under the control of Gal4-responsive UAS (upstream

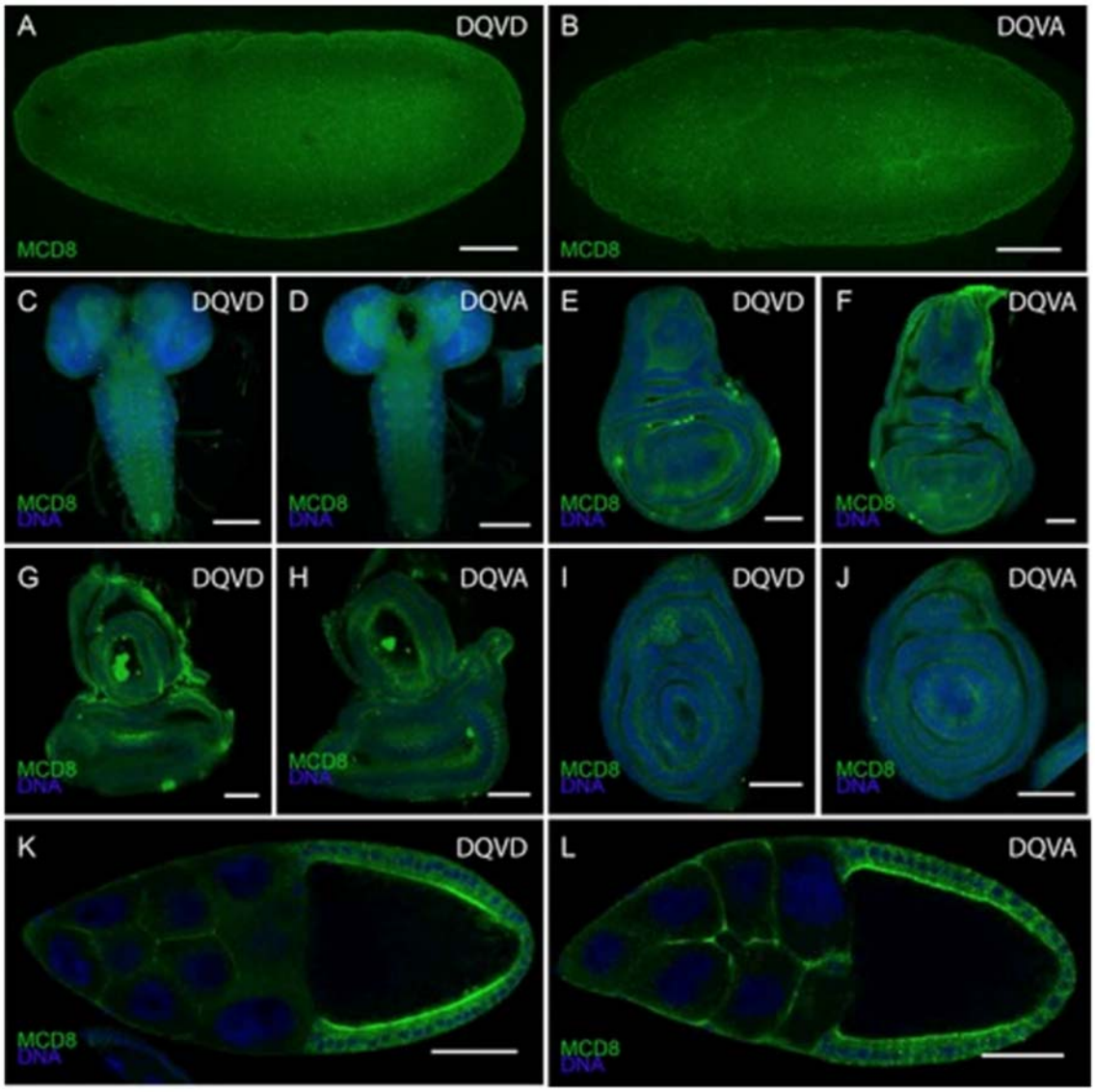

Figure 2. Expression of CasExpress DQVD and the DQVA control.

Anti mCD8 staining of the DQVD CasExpress sensor (A, C, E, G, I, K) or DQVA caspase-insensitive control (B, D, F, H, J, L) in stage 10 embryos (A, B), and 3rd instar larval central nervous system (C, D), wing disc (E, F), eye-antennal disc (G, H) and leg disc (I, J) Stage 10 egg chambers of the adult ovary (K, L). Scale bars in A–D and K, L are 100 μm, in G–J are 50 μm.

activating sequences). G-Trace flies contain three transgenes, all on the second chromosome: UAS-RFP, UAS-FLP, which encodes a yeast recombinase enzyme, and an ubi-FRT-STOP-FRT-GFP cassette where FRT stands for FLP Recombination Target sequence. Crossing the mCD8-DQVD-Gal4 sensor to G-Trace should lead to permanent GFP expression in any cell that survives transient caspase activation and in all of its progeny, in contrast to other caspase activity reporters (Bardet et al., 2008). We expected the caspase-activated Gal4 protein to be short-lived because we had observed rapid degradation of other caspase reporters (Tang et al., 2012), so we anticipated RFP would be transient and limited to the cells that activated caspase-3 but not their progeny.

We first examined adult tissues where, to our surprise, we found widespread GFP expression (Figure 1C–L). In the intestine for example, GFP was evident in the most anterior structure, the proventriculus (Figure 1C), although little RFP was evident there, suggesting that caspase had

been active earlier during development. In the midgut both RFP and GFP appeared in a partially overlapping pattern (Figure 1C,D–D”). Large nuclei corresponding to differentiated epithelial cells expressed both RFP and GFP suggesting ongoing caspase activation, whereas a subset of small progenitor cells expressed GFP but not RFP (Figure 1D–D” arrows). Visceral muscle and hindgut showed a mixture of GFP+/RFP- cells as well as some GFP+/RFP+ cells (Figure 1E–F”). The adult eye and antenna exhibited widespread nuclear GFP but only infrequent RFP (Figure 1G–H”), suggesting that caspase had been activated earlier in development either in a large fraction of cells, or in precursors that gave rise to a large fraction of adult cells; however little activation of caspase appeared to be ongoing in the adult.

In the adult central brain and nerve cord, a minority of cells expressed GFP and/or RFP (Figure 1I–J”). In the optic lobe, many but not all cells expressed GFP and/or RFP (Figure 1I–I”), whereas in body wall muscle,

nearly every cell expressed GFP (Figure 1K–K”). In the female reproductive system, every cell of the oviduct was GFP+/RFP- in every animal, whereas the majority of germline and somatic cells in egg chambers lacked FP expression (Figure 1L). Figure 1M summarizes these findings schematically. Recently a similar strategy detected similarly widespread adult cells that survived caspase-3 activity in certain previous stage of development (Tang et al., 2015).

Distinct spatial and temporal patterns of CasExpress during development

The adult expression of CasExpress suggested that caspase 3 was activated during development. To document when caspase activation first appeared, we examined embryonic and larval stages. In *Drosophila* embryos, the only tissue that activated CasExpress robustly was the salivary gland beginning at stage 12 (Figure 3A–A”). Salivary gland expression was not detected in the DQVA control, demonstrating that

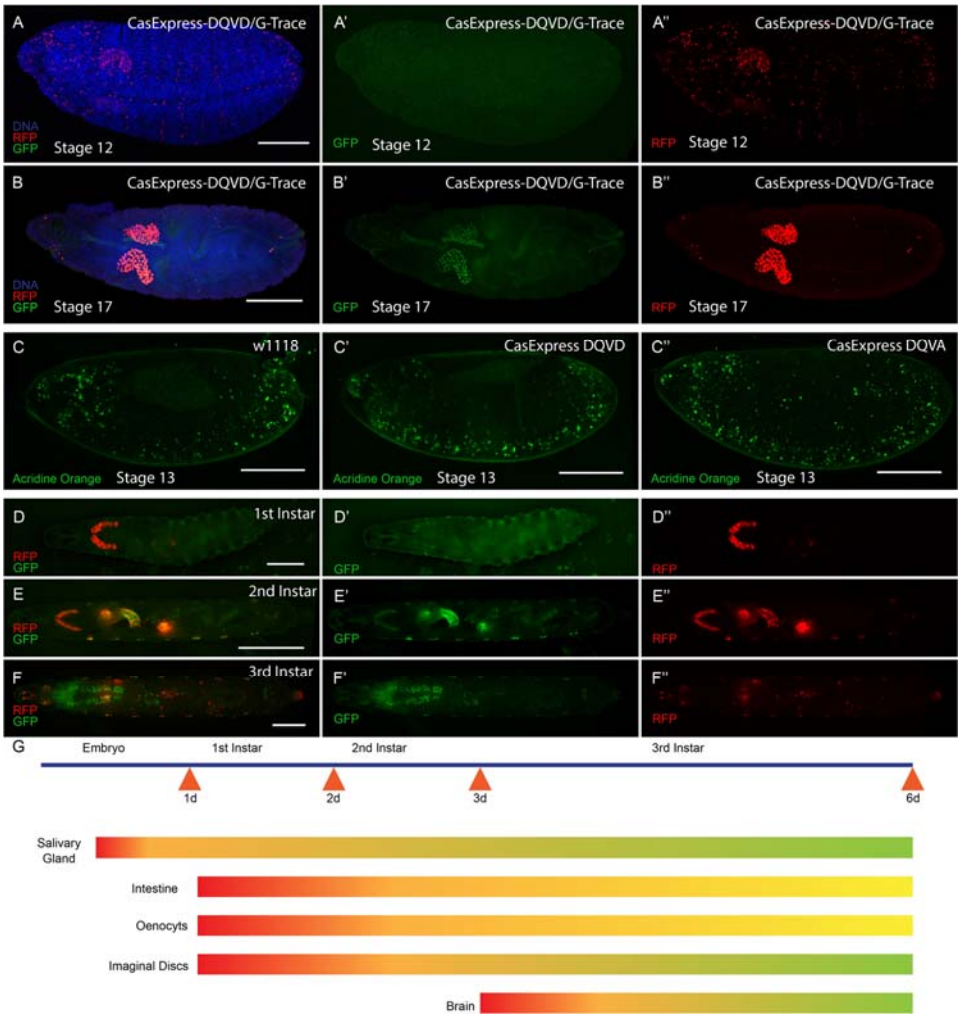

Figure 3. CasExpress activation in embryos and larvae.

(A–B") RFP and GFP expression in Drosophila embryos (A–A") stage 12, (B–B") stage 17. (C–C") Acridine orange detection of apoptotic cells in stage 13 embryos of the indicated genotypes. (D–D") 1st instar larva, (E–E") 2nd instar larva and (F–F") 3rd instar larva. (G) A schematic summarizing of GFP and RFP expression in above stages. Red represents RFP expression. Green represents GFP. Yellow/Orange indicates either a mixture of GFP positive and RFP positive cell populations or the presence of cells expressing both. Scale bars represent: 100 μm (A–C); 200 μm (D); 400 μm (E); and 600 μm (F).

this was not due to leaky or background expression from the G-Trace transgenes or random breakdown of the fusion protein that might separate Gal4 from the trans-membrane domain. In the embryo RFP was also detected in some randomly distributed cells, likely corresponding to a subset of cells that normally undergo apoptosis (Figure 3A and A”); little if any GFP was detected in those cells, presumably because dying cells did not express sufficient active caspase to transcribe and translate FLP, undergo DNA recombination, and then transcribe and translate GFP to detectable levels. While RFP was detected in the salivary gland beginning at stage 12, GFP expression became evident later (Figure 3B–B”), confirming that these fluorescent proteins exhibit different timing of activation.

Although the DQVD and DQVA proteins contained the caspase binding sequence from DIAP1 (Tenev et al., 2005), which in principle could function as a dominant-negative inhibitor of caspase activity if expressed at high enough levels, the flies expressing the sensor were viable and fertile and

showed no discernible morphological defects. The modest expression level and membrane localization presumably prevented any dominant negative effect. Moreover, there was no decrease in the number, or change in distribution, of apoptotic cells in DQVD and DQVA embryos compared to w1118 embryos (Figure 3C–C”).

Both RFP and GFP continued to be expressed throughout embryonic and larval development (Figure 3D–F”). The temporal appearance of RFP and GFP in embryonic and larval life are indicated schematically in Figure 2G.

During larval development CasExpress activation appeared over time in many cell types and tissues including all imaginal discs, oenocytes, and in subsets of neurons (Figure 3E and Figure 4). Tissues from flies carrying G-Trace in the absence of the caspase sensor or in

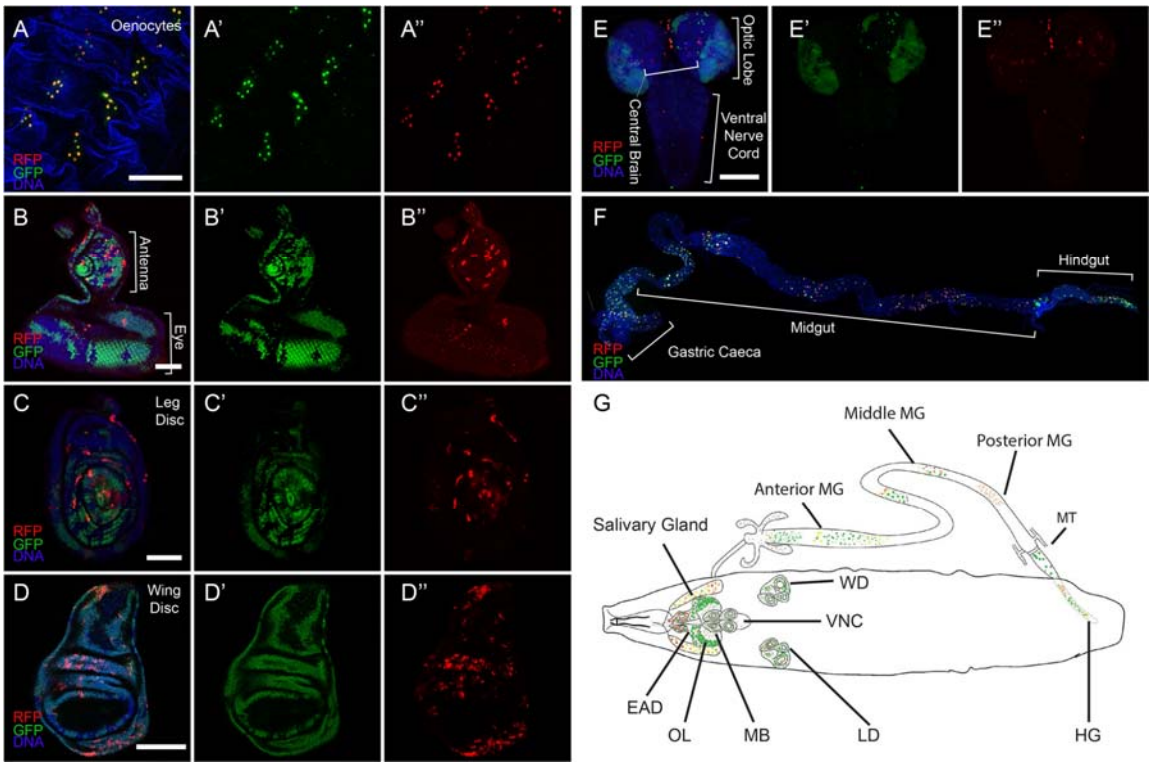

Figure 4. CasExpress activation in larval tissues.

(A–F) Confocal micrographs showing overlays of DAPI, RFP and GFP expression in the indicated tissues of wandering 3rd instar larvae. (A'–E') GFP only. (A"–E") RFP only. The brackets in D mark the eye and antenna parts of the disc, in E mark the position of optic lobe, central brain and ventral nerve cord, and in F mark the different regions of the gut. Scale bars in A and F are 200 μm, in B and C are 50 μm, in D and E are 100 μm. (G) A schematic summarizing of GFP and RFP expression in larvae. There is little GFP/RFP expression in trachea or muscles, which are not included in diagram.

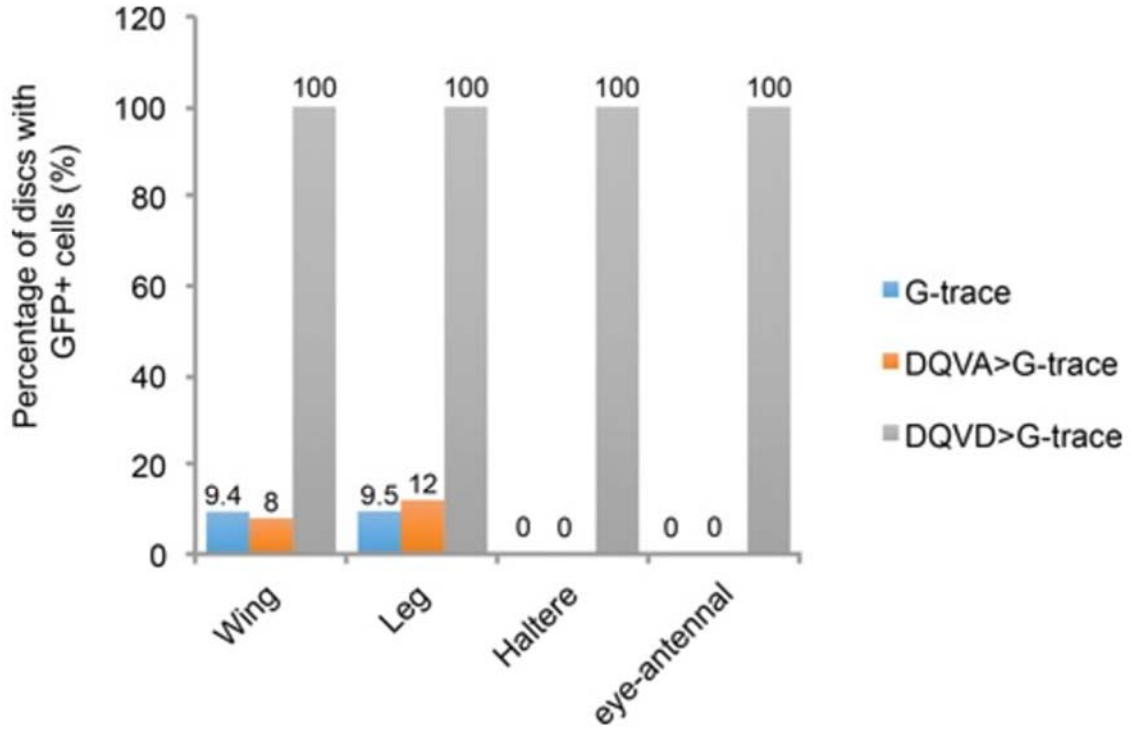

Figure 5. Comparison of GFP expression in the CasExpress DQVD sensor, the DQVA control or G-trace alone.

The percentage of wing, leg, haltere and eye-antennal discs containing GFP-expressing cells for the G-Trace alone (blue bars), G-Trace together with the DQVA caspase-insensitive control (orange bars), or G-Trace together with the DQVD caspase sensor (gray bars) are shown.

combination with the DQVA caspase-insensitive control exhibited infrequent FP expression in small clones in a minority of animals (Figure 5). The frequency and patterns were very similar regardless of the presence or absence of the DQVA control transgene (Figure 5, suggesting that this minor background was due to leaky, Gal-4-independent FLP expression from the UAS-FLP transgene. In contrast, expression in the presence of the DQVD caspase-sensitive construct was present in every animal (Figure 5), and in large fractions of cells (Figure 4).

Different tissues exhibited distinct temporal and spatial patterns of FP expression. For example oenocytes exhibited RFP and GFP expression in virtually every cell and in every animal (Figure 4A–A”). In contrast, in imaginal discs fewer cells expressed RFP as compared to GFP (Figure 4B–D”). Although every disc from every animal exhibited expression, the precise patterns varied. In the developing central nervous system (CNS) the patterns were not bilaterally symmetric. In the imaginal discs the patterns,

particularly of RFP, varied from animal to animal and did not appear to correspond to known developmental patterns of known signaling pathways or cell types.

Tissues that showed little or no activation of the sensor during normal development up through the third instar included somatic muscles, trachea, and the ventral nerve cord (Figure 4E–E”). Although most of the nervous system showed little sensor activation, a consistently large fraction (50–80%) of cells in the developing optic lobes were GFP-positive (Figure 4E–E”). In the larval intestine, partially overlapping GFP and RFP expression patterns were observed (Figure 4F), and while the overall regional patterns were conserved from one animal to the other, the details varied. The third instar larval patterns are summarized schematically in Figure 4G.

Caspase dependence of the sensor

The unexpectedly widespread activation of CasExpress raised the question as to its caspase-dependence. The sensor inserted into the attP40

site and the randomly inserted sensor demonstrated similar patterns. The absence of expression in the DQVA control demonstrated that a proteolytic cleavage at the aspartic acid was likely necessary. To address the possibility that a protease other than caspase activated CasExpress, we crossed the sensor and G-Trace into a homozygous dronc mutant background. Dronc encodes the upstream apoptotic caspase in Drosophila (equivalent to caspase-9 in mammals, (Meier et al., 2000; Hawkins et al., 2000) and its activity is necessary for activation of both fly executioner caspase molecules Drice and Dcp-1 (Florentin and Arama, 2012; Song et al., 1997; Fraser et al., 1997; Fraser and Evan, 1997; DeVorkin et al., 2014; Muro et al., 2006). Although Dronc mutants are homozygous lethal, they survive to the third instar larval stage allowing us to assess CasExpress at that stage.

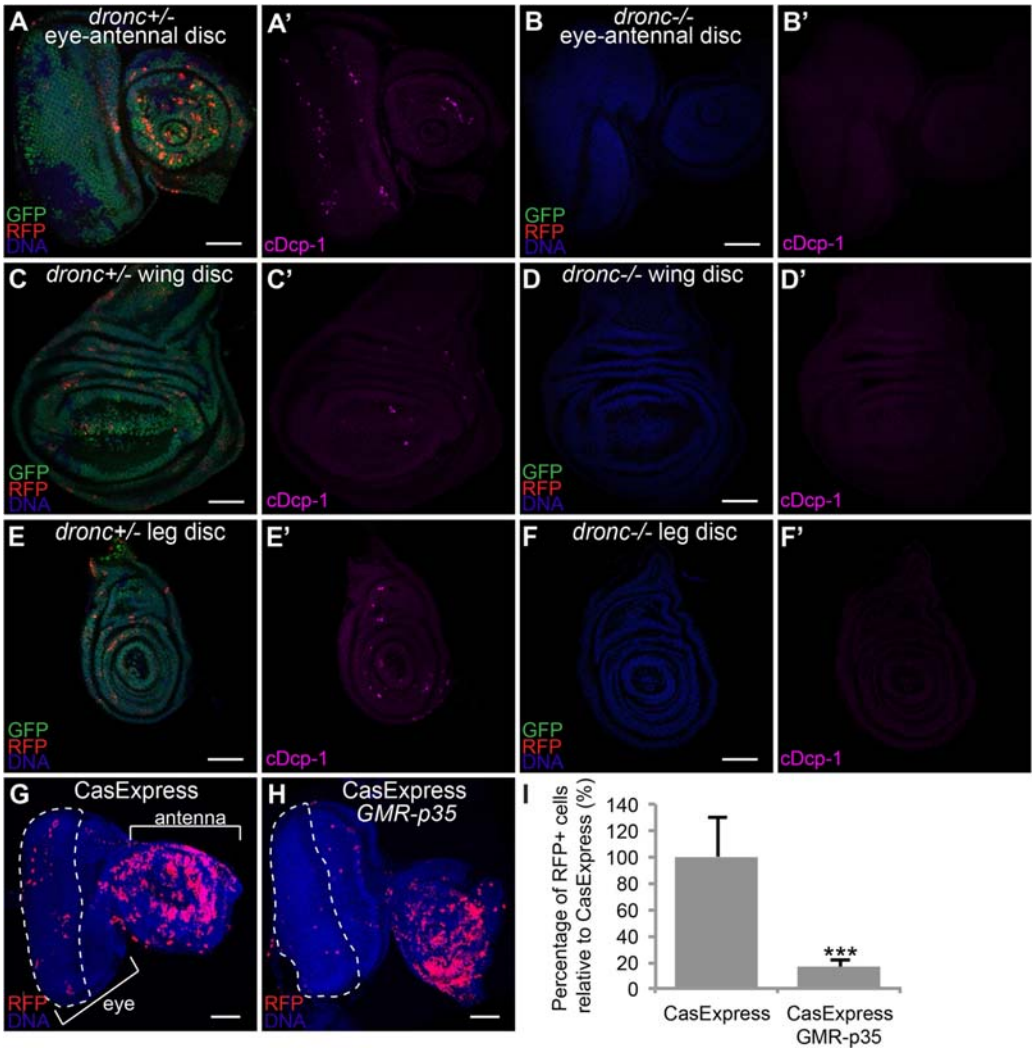

Figure 6. Caspase-dependence of CasExpress.

(A–F) Confocal micrographs showing overlays of DAPI, RFP and GFP expression in third-instar larval eye-antennal disc (A–B), wing disc (C–D), and leg disc (E–F). CasExpress and G-trace were crossed into heterozygous (A, C, E) or dronc homozygous (B, D, F) dronc mutants. (A'–E') Cleaved Dcp-1 staining of corresponding discs. Scale bars are 50 μm. (G–H) RFP expression in eye-antennal discs of late third-instar larvae with CasExpress and G-trace with (H) or without (G) GMR-p35. The dashed line encircles the region where p35 is expressed. (I) Quantification of of RFP: DAPI area. Error bars show standard error of the mean, and

As expected, the homozygous dronc mutant background eliminated caspase activity detected with an antibody against cleaved and activated Dcp-1 (c-Dcp-1) (Figure 6A–F'). The dronc mutant also eliminated virtually all RFP and GFP expression in imaginal discs from CasExpress (Figure 6A–F).

Homozygous dronc mutant embryos retained RFP and GFP expression in the salivary gland, possibly due to the persistence of maternal caspase expression. To confirm the presence of cleaved caspase in embryonic salivary gland cells, which has not been previously reported, we stained CasExpress embryos with an antibody against cleaved caspase-3 (Figure 7). Despite the absence of other apoptotic markers in these cells, salivary glands did label with this antibody, suggesting a non-apoptotic function for caspase in this tissue.

The Baculovirus p35 protein inhibits both executioner caspases DrIce and Dcp-1, but not Dronc (Meier et al., 2000). Therefore we

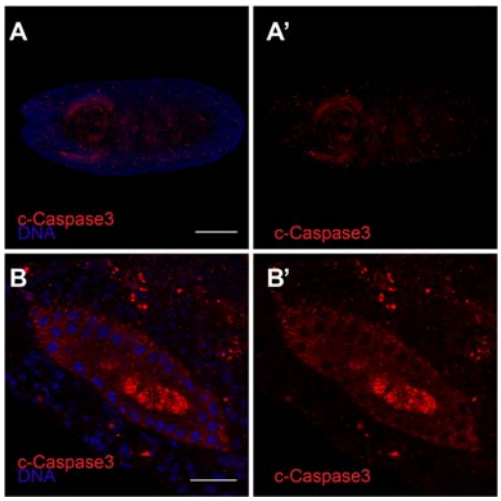

Figure 7. Anti-cleaved caspase-3 (red) and DAPI (blue) staining of stage 14 embryo (A) and high magnification of a salivary gland (B).

(A'–B') are cleaved caspase-3 only. Scale bar in A is 20 μm, in B is 100 μm.

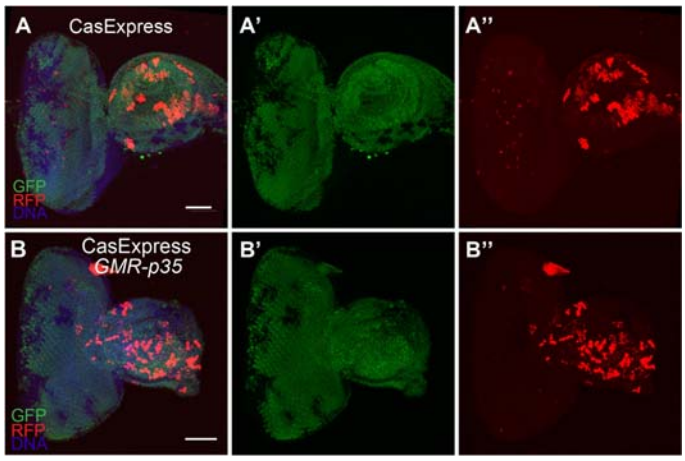

Figure 8. GFP and RFP expression of CasExpress in a wild type eye-antennal disc (A–A"), or one carrying the GMR-p35 transgene (B–B").

Scale bars are 100 μm.

crossed GMR-p35, which is a transgene that expresses p35 in the eye imaginal disc posterior to the morphogenetic furrow (Hay et al., 1994), into the CasExpress/G-Trace flies. GMR-p35 significantly reduced the number of RFP+ cells in the posterior eye disc compared to the control (Figure 6 G-I), whereas no change in RFP was observed in the antennal disc, which served as an additional internal control. The few remaining RFP+ cells in the posterior eye disc likely were cells that had activated Gal4 prior to the onset of expression of the GMR promoter. GFP expression was still evident, indicating that caspase activation preceded expression of p35 from the GMR enhancer/promoter in those cells (Figure 8).

One known non-apoptotic role for caspase activity is in the innate immune response. Specifically the upstream caspase Dredd activates NFkB signaling and expression of anti-microbial peptides (Meinander et al., 2012; Leulier et al., 2000). The gut is known to have a highly active innate immune response. Therefore to determine whether the CasExpress activity we

detected in the gut was related to the immune response, we crossed CasExpress into dredd mutant animals. However we detected no difference in the GFP or RFP expression level or pattern between dredd mutants and heterozygous wild type siblings, in any tissue examined (Figure 9).

Discussion

Here we report the first systematic analysis of the fates of cells that survive caspase-3 activation throughout Drosophila development. The most striking results of this study include widespread cell survival of caspase activation and the distinct spatial and temporal patterns observed in different tissues throughout development. Caspase-3 activation has been strongly associated with cell death (Thornberry, 1998; Chipuk et al., 2006). While some previous studies have indicated that caspase-3 can perform other functions (e.g.[Connolly et al., 2014]), these

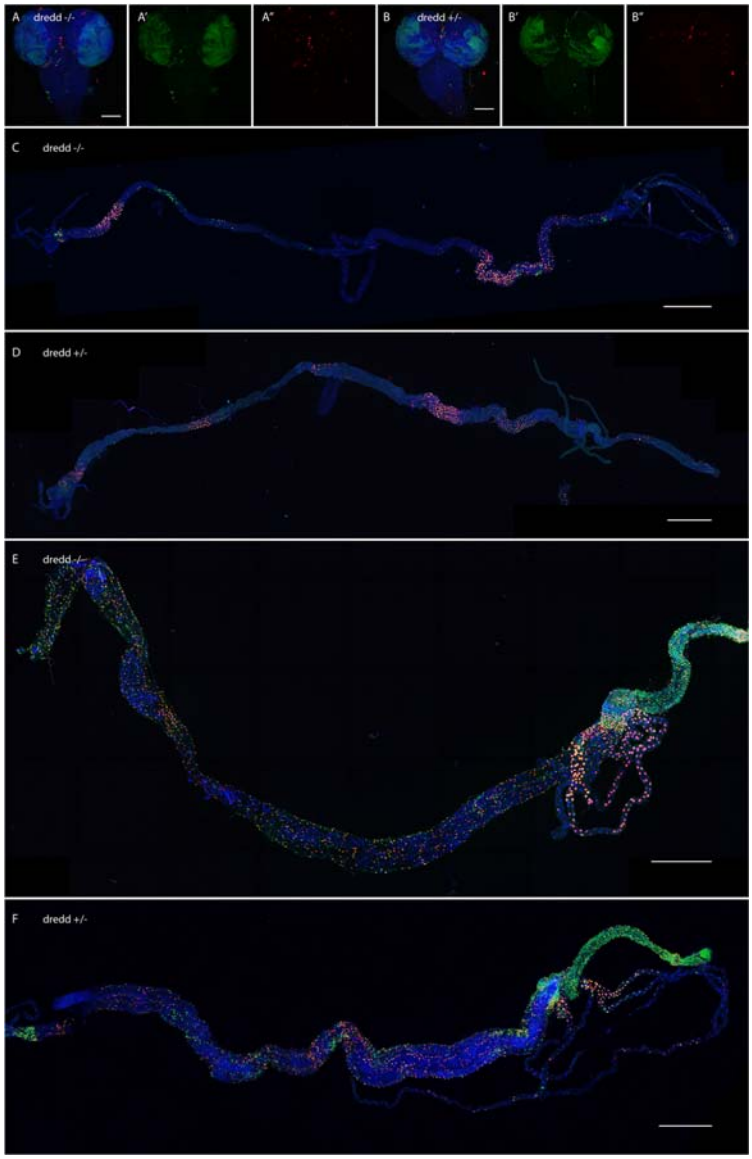

Figure 9. Loss of dredd does not change CasExpress patterns.

(A–F) Confocal micrographs showing overlays of DAPI, RFP and GFP expression in third-instar larval CNS (A–B), larval intestines (C–D), and adult intestines (E–F). CasExpress and G-trace were crossed into heterozygous (A, C, E) or homozygous (B, D, F) dredd mutants. (A'–B') GFP only, (A''–B'') RFP only. Scale bars in A and B are 100μm, in C and D are 800μm, in E and F are 400μm

non-apoptotic caspase activities are generally considered exceptions to the rule. However it has not been possible to systematically follow the fates of cells that experience executioner caspase activity throughout development in vivo.

We observed several distinct patterns of CasExpress activation, which likely reflect different biological functions. Oenocytes and cells of the salivary gland and Malpighian tubules exhibited activated CasExpress in every cell, in every animal, with no evidence of apoptosis or even partial cellular destruction. This pattern seems most consistent with non-apoptotic roles for caspases. One known protein target of Drice and Dcp1 that might be relevant in this context is the Sterol Regulatory Element-binding Protein (dSREBP) (Amarneh et al., 2009). SREBP is synthesized as a membrane-tethered precursor that is released by proteolytic cleavage so that it can translocate to the nucleus where it transcribes target genes involved in lipid synthesis and uptake. Oenocytes have an established role

in lipid synthesis (Makki et al., 2014). According to FlyBase, SREBP is expressed at high levels in many of the tissues that show constitutive caspase-3 activity. SREBPs in mammals and flies are cleaved by site-2 protease (S2P). However Drice and Dcp1 can also cleave SREBP and can even substitute in the absence of S2P in Drosophila (Amarneh et al., 2009). It is possible then that one function of caspase activity in oenocytes and other cell types is to activate SREBP during normal development and/or in times of stress to meet metabolic demands.

The published literature provides clear examples of caspase activities that promote cellular remodeling via limited destruction such as sperm maturation (Arama et al., 2003; Huh et al., 2004), remodeling of neurites (Yan et al., 2001; Finckbone et al., 2009), and enucleation of certain terminally differentiating cells, such as erythrocytes and lens epithelial cells (Connolly et al., 2014). In such tissues one would also expect to see CasExpress activated in a reproducible temporal and spatial pattern.

Metamorphosis is a period of insect development during which extensive tissue remodeling occurs. The CasExpress activity that we detected during pupal life in the nervous system for example could be a consequence of remodeling.

In contrast, in imaginal discs and the brain CasExpress was activated sporadically in both space and time, during periods of development when apoptosis is known to occur. This pattern seems most consistent with developmental anastasis. Anastasis was first described in cultured cells and is defined as the recovery of cells from the brink of apoptotic cell death after caspase-3 activation (Tang et al., 2012). During normal development of many tissues including Drosophila imaginal discs and mammalian blastocysts, cells are thought to compete for survival based on differential fitness (Moreno and Rhiner, 2014; Merino et al., 2015; Kolahgar et al., 2015; Vincent et al., 2013; de Beco et al., 2012). Differences in fitness can be detected amongst cells with artificially induced differences in growth rates,

caused by differential ribosomal protein levels (Morata and Ripoll, 1975), c-Myc expression levels (de la Cova et al., 2004), or access to trophic factors (de Beco et al., 2012). In the nervous system for example, those cells that obtain sufficient growth factor signaling survive and those that receive too little undergo apoptosis. This has generally been considered an all-or-nothing decision. However the observations that cultured cells exposed transiently to a lethal toxic stimulus can recover after caspase-3 activation, and the survival of cells exposed to a sublethal dose of radiation or mitochondrial permeabilization (Liu et al., 2015; Ichim et al., 2015), raise the question as to whether cells might actually bounce back from transient caspase activation during development as well. Our results show that cells can survive caspase-3 activation during normal development, perhaps due to recovery from a transient apoptotic stimulus.

Another possibility is that cells in a population differ in their sensitivities to apoptosis due to variation in epigenetic states (Flusberg and

Sorger, 2015; Spencer and Sorger, 2011). In mammals the E3 ubiquitin ligase PARC can target cytoplasmic cytochrome c for ubiquitin-mediated degradation, providing one molecular mechanism by which cells can recover from an apoptotic stimulus (Gama et al., 2014). Differential expression of PARC may confer different levels of resistance to executioner caspase activity. Other, as yet unknown, epigenetic differences between cells may also confer differing sensitivities to caspase activation.

Materials and methods

Fly strains

The following transgenic and mutant strains were used:

The CasExpress biosensor (pattB-Ubi-CasExpress-DQVD) and caspase-insensitive control (pattB-Ubi-CasExpress-DQVA) were newly generated as follows. First, a backbone pattB-synaptobrevin-7-QFBDAD-hsp70 (gift from Christopher J. Potter lab) was linearized with restriction enzymes AatII and BamHI. The poly-ubiquitin promoter was cloned by PCR from pUWR (Addgene, Cambridge, MA), and the product was inserted to backbone by In-Fusion Cloning Kit (Clontech Laboratories, Mountain View, CA). The product, which was verified by sequencing was named pattB-Ubi. Second, pattB-Ubi was linearized with restriction enzymes NdeI and PstI as a backbone. An insert consisting of the sequence of MCD8, DIAP1 (residues 2–147) and Gal4 in 5’ to 3’ order and two 15 bp sequences overlap with backbone on both 3’ and 5’ end was generated by PCR and In-

Fusion cloning. Residues 21 and 22, immediately following the DQVD cleavage site in DIAP1, were mutated from sequence NN to GV, in order to protect the cleaved product from possible N-end rule degradation. Third, the insert and backbone were ligated using the In-Fusion kit. A product verified by sequencing was named pattB-Ubi-CasExpress-DQVDGV. Finally, nucleotide 59 of DIAP1 sequence in pattB-Ubi-CasExpress was mutated to change amino acid 20 from D to A by single point mutagenesis. A product verified by sequencing was named pattB-Ubi-CasExpress-DQVAGV. CasExpress and control plasmids were sent to BestGene Inc., inserted to Perrimon strain attP40 through a PhiC31 integrase mediated transgenesis. Random insertions of CasExpress-DQVDNN and CasExpress-DQVANN were also generated. DroncI29 was a gift from Kenneth D. Irvine. The following strains were obtained from the Bloomington Stock Center: G-Trace (Bloomington #28280); tub-Gal80ts (Bloomington #7018); GMR-p35 (Bloomington #5774); UAS-p35 BH1 and BH2 (Bloomington #5072 and

5073). All lines and crosses were kept at 25°C except where otherwise indicated.

Dissection, immunohistochemistry and imaging

Larval intestines, oenocytes (together with surrounding cuticle) and adult muscles, brains, eyes, ovaries, oviducts, uteri, tissues were dissected in PBS. For adult ventral nerve cords, whole thoraxes were used for fixation. For larval tissues, the anterior 1/3 part of larvae was cut off and turned inside out, all tissues remained attached to cuticle during fixation. Tissues were fixed in 4% paraformaldehyde in PBS at room temperature for 10 min (larval cuticles with CNS and imaginal discs) 30 min (adult thoraces). Other tissues were fixed for 15 min. After fixation, adult ventral nerve cords were dissected from adult thoraces. The samples were then washed with PBS/0.3% Triton X-100 (PBSt) for 3 x 10 min and blocked with 5% goat serum for 30 min. Fluorescence of RFP and GFP were detected directly without antibody staining. Mouse anti-mCD8 (Santa Cruz, Dallas, TX,

#51735, 1:50), and rabbit anti-Cleaved Dcp-1(Asp216) (Cell Signaling, Danvers, MA, #9578, 1:100) were incubated with dissected tissues overnight at 4°C, followed by 3 x 10 min PBSt washing and secondary antibody incubation for 2 hr at room temperature. Samples then were washed twice for 15 min each with PBSt and incubated for 15 min with 10 ng/ml Hoechst 33,342 in PBSt. After Hoechst staining, larval CNS and imaginal discs were dissected away from cuticle. All samples were mounted in Vectashield mounting media (Vector Laboratories, Burlingame, CA, H-1000).

A Zeiss AxioZoom microscope was used for imaging whole larvae.

A Zeiss LSM 780 confocal microscope was used for the rest of images. Embryo collections, fixation and acridine orange (Sigma-Aldrich, St. Louis, MO, A6014) staining of embryos are as described (McCall and Peterson, 2004)

Image analysis and quantification

Images were processed with Fiji. A threshold for each channel of interest (COI, e.g. RFP, GFP) was set by auto-threshold (method: Default, Dark). For anti-cDcp1 the threshold was set using MaxEntropy. Area above threshold was measured as S[COI]. The area of DNA was measure in the same manner as S[DNA]. A ratio of S[COI]/S[DNA] was then calculated.

GMR-p35 suppression of CasExpress in antennal-eye disc

To test suppression of CasExpress by the caspae inhibitor p35, larvae with the genotype GMR-p35; CasExpress/G-Trace; TubGal80ts were raised in 18°C until early third instar. Larvae were then transferred to 29°C and incubated for 48 hr. Antennal-eye discs were then dissected, fixed and stained with Hoechst 33342, followed by a Z-stack imaging on LSM780 microscope (Objective: 20x Zeiss plan-apochromat dry, 0.8 NA; step-size: 1.46 μm).

Images were process with Fiji. A Z-projection of each image was generated by maximum intensity algorithm. An ROI was drawn to define

the boundary of the antennal disc. A threshold for the RFP channel was determined by auto-threshold (method: Default, Dark). Another ROI was then drawn to define the boundary of region of the eye disc posterior to the morphogenetic furrow. Threshold of RFP channel of original image without Z-projection was set as (0.5a, b). Area of RFP above threshold was measured for each layer and summed. Area of DNA above threshold determined by auto-threshold (method: Default, Dark) was also measured for each layer and summed. The ratio of summed areas of RFP and DNA was then calculated.

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Chapter II

The Temporal and spatial features and functions of CasExpress signals in Drosophila Melanogaster

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Abstract

CasExpress is a lineage tracing biosensor that temporarily and permanently marks cells in Drosophila, which have undergone Caspase activation, but have subsequently survived, with fluorescent protein expression. We have previously shown that CasExpress reveals widespread survival of caspase-3 activity in Drosophila larvae and adults. Here we characterize the spatial and temporal patterns of CasExpress that emerged in different tissues. Some cells activated caspase-3 during their normal development in every cell and in every animal without evidence of apoptosis. In other tissues, such as the brain, expression was sporadic both temporally and spatially and overlapped with periods of apoptosis. In adults, reporter expression was evident in a large fraction of cells in most tissues of every animal; however the precise patterns varied. Inhibition of caspase activity in wing discs reduced wing size demonstrating functional significance. The implications of these patterns are discussed.

Introduction

Apoptosis is important to maintain the normal development and homeostasis of multicellular organisms. Apoptosis can be induced by a variety of either intrinsic or extrinsic stimuli. Upon induction of apoptosis, different pathways converge to the activation of a family of protease enzymes called caspases. Caspases then cleave a variety of downstream targets, thereby triggering programmed cell death.

Although the activation of caspase usually causes programmed cell death, survival from caspase activity has also been observed in different occasions. In *Drosophila melanogaster*, we designed a biosensor named CasExpress that has revealed widespread survival of caspase-3 activation. This striking result led us to further characterize the temporal and spatial patterns of the cells that survived caspase-3 activity, as well as the functions of this survival process in development.

Utilizing Gal80TS, a temperature sensitive inhibitor of Gal4, which enables us to only let CasExpress be activated during certain stages of *Drosophila* development, we observed distinct categories of CasExpress activation. For example, in some organs, every cell activated the sensor over an extended period of development without evidence of apoptosis or morphological remodeling, suggesting a function for caspase-3 unrelated to cellular destruction. In other tissues, activation was sporadic in temporal and spatial pattern, suggesting a stochastic process. In these tissues, the precise patterns differed from animal to animal, and occurred in regions that normally exhibit apoptosis. These observations suggest that some cells recover from the brink of apoptotic cell death and undergo developmental anastasis. We propose that these different patterns represent distinct functions of executioner caspases during normal development.

Result

To address when the CasExpress was activated in various tissues, we silenced CasExpress during most of development, by crossing in the temperature-sensitive (ts) version of Gal80 ($Gal80^{ts}$), which represses expression from UAS transgenes even in the presence of Gal4. When flies carrying CasExpress, $Gal80^{ts}$, and G-TRACE were grown at 18°C, GFP was completely repressed, and even the infrequent, random clones due to leaky expression of UAS-FLP was suppressed (Fig 1).

Imaginal disc CasExpress arises sporadically in time

The large percentage of GFP+ cells in late third instar larval tissues raised the question as to whether caspase was activated in a significant fraction of cells at one particular stage in development, or alternatively whether caspase was activated sporadically in time. To address this question, we grew Gal80ts/CasExpress/G-Trace flies at 18°C and then shifted them to 29°C for 24 hr either at the first instar (Figure 2, upper

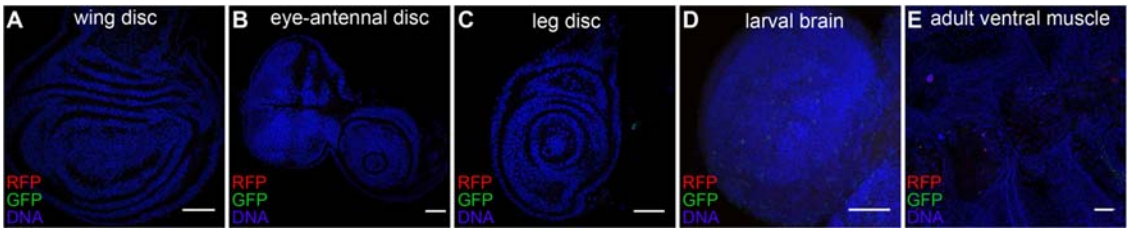

Figure 1. GFP, RFP and DAPI fluorescence in imaginal discs from flies carrying CasExpress, Gal80ts, and G-TRACE raised at 18°C.

GFP and RFP are completely repressed.

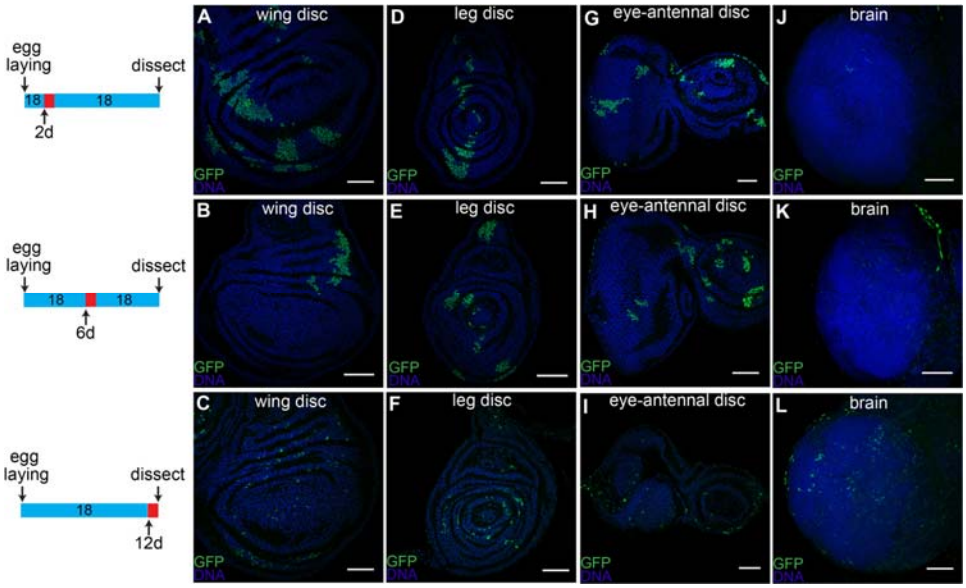

Figure 2. Timing of caspase activation in larval tissues.

Larvae with CasExpress, G-trace and Gal80ts were grown at 18°C (blue in the timeline bars on the left) for 2d (A, D, G, J), 6d (B, E, H, K), or 12d (C, F, I, L), shifted to 29°C (red in the timeline bars) for 1d, then kept at 18°C until late third instar. Induction of GFP expression occurs in wing discs (A–C), leg discs (D–F) and eye-antennal discs (G–I) throughout the larval stage; whereas few cells in brain (J–L) survive caspase activation before third instar. Scale bars are 50 μm.

panels), the second instar (Figure 2, middle panels) or the mid third instar (Figure 2, lower panels). We then returned them to 18°C and dissected them at the late third instar larval stage. Rather than all the GFP+ cells arising at one particular stage, sporadic expression was observed regardless of when the temperature shift occurred. This was true in wing (Figure 2A–C), leg (Figure 2D–F) and eye-antennal discs (Figure 2G–I). Cells that activated the sensor later produced smaller patches of cells, as expected if the patches represent clonal descendants of a single event. However we cannot rule out the possibility that separated cells that activated the sensor subsequently coalesced into patches based on differential adhesion. In contrast to the discs, caspase activation in the brain was limited to late larval stages (Figure 2J–L).

The temporal and spatial pattern of brain CasExpress

Apoptosis plays a particularly important role in the nervous system, and we detected cleaved caspase throughout larval CNS (CNS)

development, both in w1118 and in DQVD sensor flies (Figure 3). Therefore we characterized the temporal and spatial activation of CasExpress in this tissue in more detail. We combined CasExpress, G-Trace and Gal80ts. Flies kept at 18°C to silence CasExpress throughout development exhibited virtually no expression of RFP or GFP. However if we shifted them to 29°C for 12 hr at the early (Figure 4A), middle (Figure 4B), or late (Figure 4C,D) third instar and dissected them near the end of larval development, we observed GFP expression in seemingly random locations. Similar numbers of GFP-expressing cells appeared regardless of precise developmental stage. Figure 4E–H shows the patterns observed in 10 different animals, each in a different color, demonstrating the variability. The patterns were clearly not bilaterally symmetrical. We conclude that cells survive caspase activation sporadically during CNS development. This pattern seems more consistent with that expected for developmental anastasis, that is

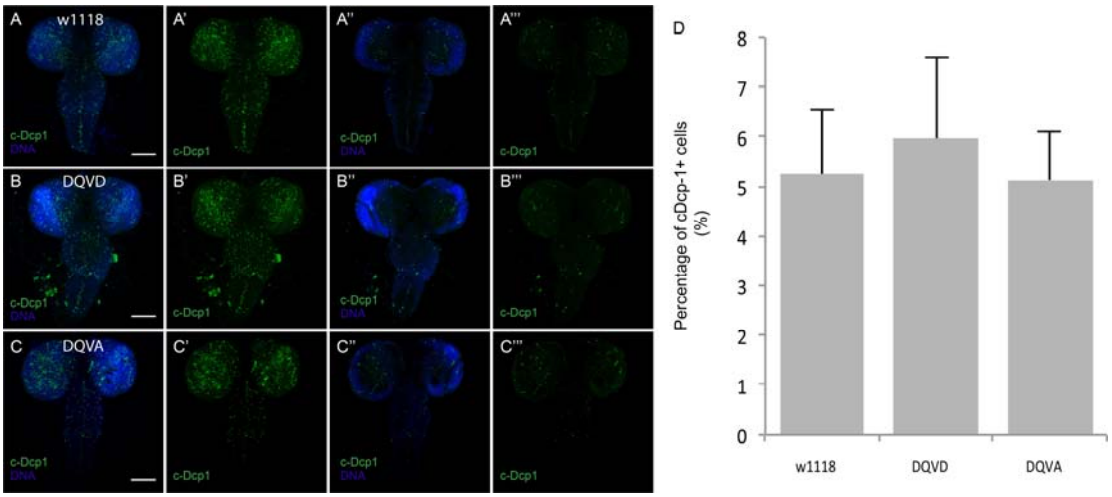

Figure 3. Caspase activity during larval CNS development.

(A,B,C) Anti-cleaved Dcp1 antibody staining (green) detects caspase activity during larval brain development in w1118 (A) CasExpress DQVD (B) and DQVA control (C). (A–C) (A'–C') are Z-projection of images generated by maximum intensity algorithm, (A''–C'') (A'''–C''') are single slices of each image. (A'–C') (A'''–C''') are cleaved Dcp1 only. (D) Quantification of the percentage of cDcp-1-expressing cells. Scale bars are 100μm.

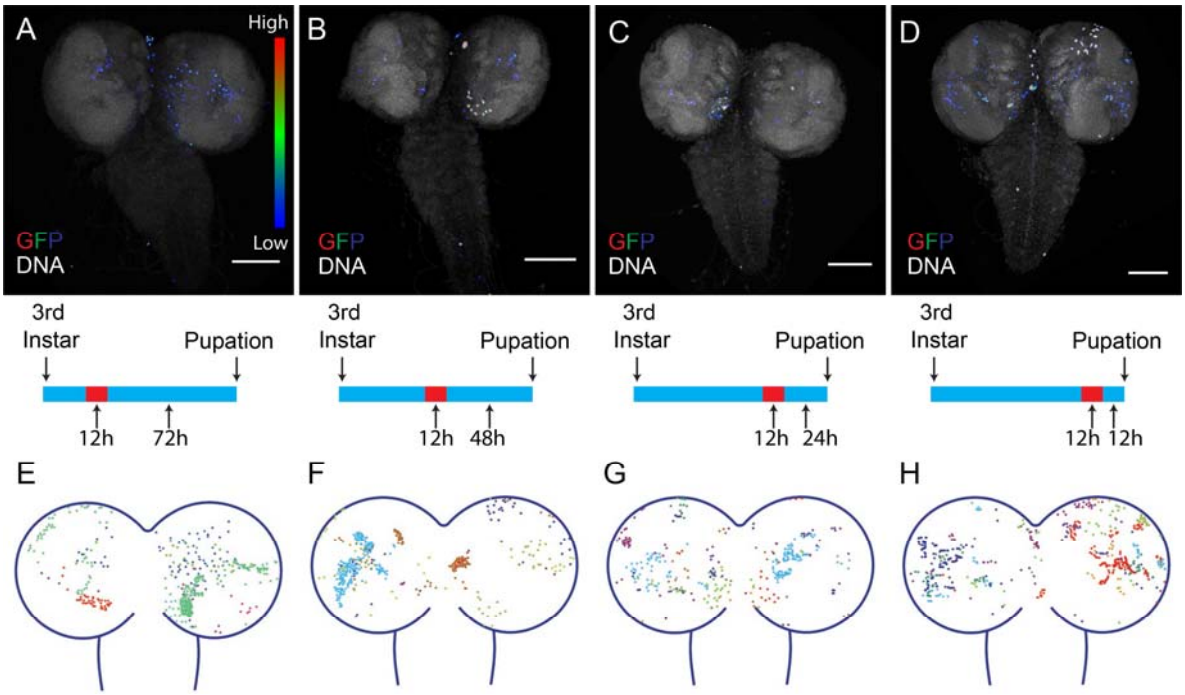

Figure 4. Timing of CasExpress activation in larval CNS.

Larvae with CasExpress G-Trace and Gal80ts were grown in 18°C (blue bars in the middle) until 3rd instar and shifted to 29°C for 12 hr. Then larvae were kept at 18°C for 72 hr (A, E), 48 hr (B, F), 24 hr (C, G) or 12 hr (D, H) until they reached late 3rd instar. (A–D) Four examples of GFP expression patterns in the larval CNS presented in Rainbow RGB, which shows different levels of GFP intensity in different colors. (E–H) Z-projections of different samples were slightly transformed and fit into the diagram of brain. The positions of GFP positive cells for each sample are indicated with different colors. Scale bars are 100 μm.

recovery from the brink of apoptotic cell death, rather than a precise role for caspase in the development of a specific cell type.

CasExpress activation during metamorphosis

Metamorphosis requires remodeling of some tissues and wholesale destruction and rebuilding of others (Baehrecke, 2002; Yu & Schuldiner, 2014). Apoptosis contributes substantially to these processes. To address how much of the adult expression arose during metamorphosis in each tissue, we crossed in the Gal80ts repressor and grew flies at 18°C to prevent the induction of CasExpress. We then shifted the flies to 29°C, the non-permissive temperature for Gal80ts, to allow induction only during specific time windows corresponding to larval, pupal, or adult stages respectively (Figure 5A). Distinct patterns were observed in different tissues. In the antenna (Figure 5B–D") and brain (Figure 5E–G"), CasExpress was activated during larval and pupal stages but virtually none was detected in adulthood. Activation during

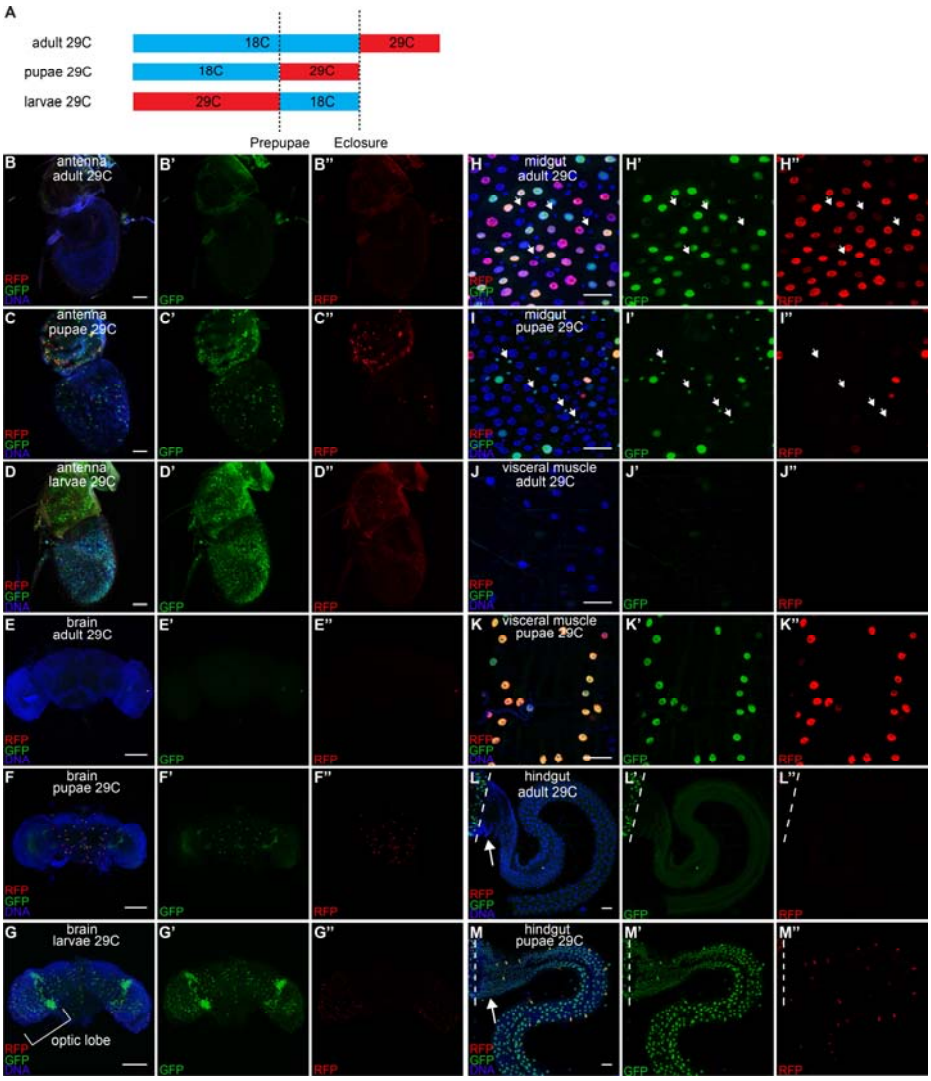

Figure 5.

Developmental timing of caspase activation in adult tissues.

(A) A schematic of the timing of the temperature shifts (blue: 18°C, red: 29°C) during the growth of flies with CasExpress, G-trace, and Gal80ts. (B–M) GFP and RFP expression in antenna (B–D), brain (E–G), midgut (H–I), visceral muscle surrounding midgut (J–K), and hindgut (L–M) in flies with CasExpress, G-trace, and Gal80ts that grown at the condition indicated in the panels. Panels marked with prime showed separated channels of the left. Arrows in H–I'' point to some examples of GFP+ progenitor cells. Dotted lines in L–M'' mark the boundary between midgut and hindgut. Arrows in L and M point to the hindgut proliferation zone. Scale bars in B–D, and H–M are 25 μm. Scale bars in E–G are 100 μm.

the pupal period could be responsible for remodeling of connections during metamorphosis and was not unexpected, however the more extensive activity during the larval period suggests an additional function for caspase in earlier nervous system development. In midgut enterocytes, some activation occurred during pupal life but more appeared in the adult (Figure 5H–I”), possibly related to the biology of midgut enterocytes which face damage and undergo rapid turnover in adults even under normal physiological conditions. In visceral muscle surrounding the midgut (Figure 5J–K”) and in the hindgut (Figure 5L–M”) activation was limited to pupal stages, consistent with a role for caspase in metamorphosis of this tissue.

When CasExpress induction was allowed only during the pupal stage, some progenitor cells in the midgut (Figure 5I–I’’), visceral muscle surrounding it (Figure 5K–K’’), and the whole hindgut including the proliferation zone, which contains progenitor cells (Figure 5M–M’’),

showed GFP expression. Thus caspase was activated during metamorphosis and some cells survived this event. This is intriguing because during metamorphosis the larval gut degenerates and the adult gut is reconstituted by progenitor cells (Micchelli, 2012). We did not detect RFP in progenitor cells at any stage that we analyzed, and we only detected GFP in progenitors when CasExpress was allowed to be active during the pupal stage. Therefore caspase is likely activated for only a brief period during pupal life. The progenitor cells, like the rest of the animal, are exposed to apoptotic stimuli such as systemic ecdysone (Jiang, Baehrecke, & Thummel, 1997), yet they survive to reconstitute the adult gut. They might survive either because they are particularly resistant to caspase activity and apoptosis, as is postulated for stem/progenitor cells generally. Alternatively caspases may actually promote their proliferation or maintenance as has been described for some mammalian progenitors (Li et al., 2010; Yoneyama, Shiba, Yamaguchi, & Ogita, 2014);

or some progenitor cells may undergo anastasis and recover from the brink of apoptotic cell death. Although we cannot currently distinguish definitively between these possibilities, the observation that a subset of progenitor cells activates CasExpress might indicate that some cells resist the apoptotic stimulus prior to activation of caspase-3 whereas others experience caspase activity and recover from it. The observation that pupal visceral muscle cells exhibit RFP and GFP in nearly every cell suggests prolonged caspase activation. Together these observations demonstrate that survival of caspase activation occurs in distinct spatial and temporal patterns among different cell types and tissues, possibly due to differing epigenetic states, developmental mechanisms, and/or physiological functions.

Functional significance of developmental caspase in the wing

We wondered if the observed caspase activity was functionally significant. The homozygous Drice mutant is lethal, as are dronc mutants.

One interpretation of this lethality phenotype is that these mutations prevent apoptosis, and that apoptosis is essential. Our results suggest an additional possibility, which is that non-apoptotic caspase activity may be important during normal development. To test this, we crossed two different UAS-p35 transgenes to the rotund-Gal4 (rn-Gal4) line, which expresses in the pouch region of the wing imaginal disc, the region that gives rise to the adult wing blade. We then evaluated the morphology and size of the adult wing for defects in growth and/or patterning. Although the wings appeared normally patterned, they showed a small (10%) but reproducible and significant reduction in area (Figure 6A–D), demonstrating the functional importance of caspase activity in this tissue. We repeated this experiment using engrailed-Gal4, which drives expression only in the posterior compartment of the wing, and compared the area of the posterior compartment to that of the anterior compartment as an internal control. Again, inhibition of

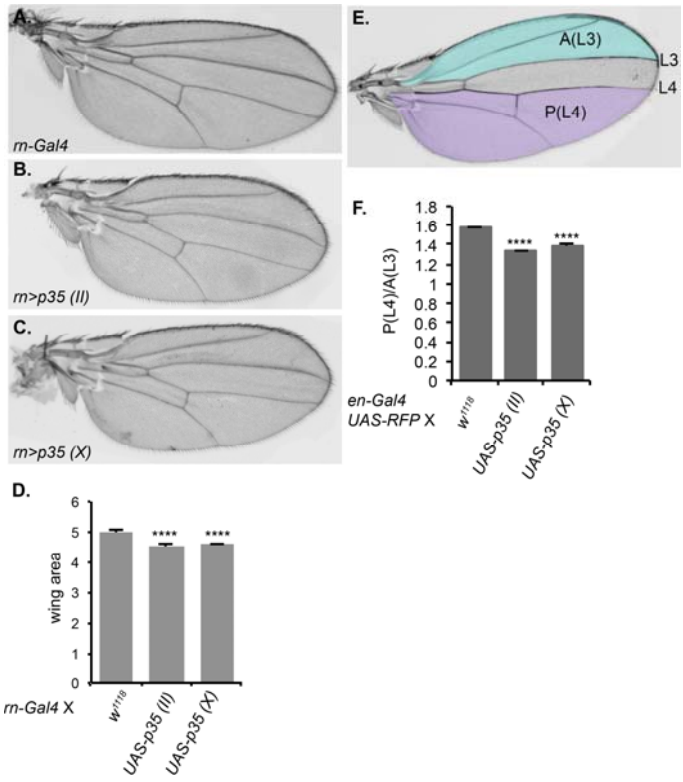

Figure 6.

Inhibition of caspase activity reduces wing size.

(A–C) Representative wings from progeny of rn-Gal4 crossed to (A) control w1118, (B) UAS-p35 on chromosome II, or (C) UAS-p35 on the X chromosome. (D) Quantification of wing area in arbitrary units. (E) Schematic showing the regions used for area measurement in wings with or without p35 expressed under en-Gal4. In the anterior compartment, we measured the area anterior to L3 vein, which is highlighted in blue and marked as A(L3). In posterior compartment, we measured the area posterior to L4, which is highlighted in purple and marked as P(L4). (F) Quantification of the ratio between P(L4) and A(L3) in wings from progeny of en-Gal4 crossed to w1118, UAS-p35 on the second chromosome,

apoptosis by expression of p35 caused a small but significant reduction in size. If the only function of caspase were to promote apoptosis, inhibition of caspase should result in excess cells, and therefore a larger size. The observation of a smaller wing suggests a different function for caspase in this tissue.

Discussion

Our results suggest that the ability of cells to survive caspase activation changes during development. Many cells in the embryo activate caspase-3, yet we detected no GFP expression in embryos, except in the salivary gland. Our temperature shift experiments revealed that, in imaginal discs for example, more and more cells survived as development progressed, and cells that activated the sensor in early stages produced large numbers of progeny such that by the end of the third larval instar, the majority of cells expressed GFP.

A number of studies have reported immunoreactivity against cleaved caspase-3 in neurons that appeared to be dividing, differentiating or migrating (Finckbone, Oomman, Strahlendorf, & Strahlendorf, 2009; Schoenmann et al., 2010; Yan et al., 2001; Yu & Schuldiner, 2014). While tantalizing, the studies were carried out in fixed tissue so the ultimate fates of such cells could not be determined. In the current study we were

able to follow the fates of cells that survived caspase activation, and these results demonstrated that in many tissues of the adult the majority of cells arise from cells that experience transient caspase activity at some point during their development. Therefore such events are not the exception; rather they are the rule.

Autonomous versus non-autonomous survival

When extra apoptosis is artificially induced in Drosophila imaginal disc cells, it stimulates surviving cells to proliferate (Fan & Bergmann, 2008). Dying cells secrete growth factors to facilitate the survival and proliferation of their neighbors in the process known as compensatory cell proliferation (Kuranaga et al., 2011; Xing, Su, & Ruohola-Baker, 2015). The marking system that we report here demonstrates cell autonomous survival of caspase activation. Both autonomous and non-autonomous survival and proliferation may cooperate to promote recovery of tissues from insults that kill some but not all cells.

A role for caspases in injury repair and tissue regeneration has been demonstrated in Hydra, Xenopus, planaria, newts and in mouse liver (reviewed in (Connolly, Jager, & Fearnhead, 2014)), indicating that this is a well-conserved and general phenomenon. Our observation that the majority of cells in the adult fly descend from cells that survive caspase activation at some point suggests that, in addition to the well-documented compensatory proliferation in response to injury, there may autonomous compensatory proliferation in cells that survive caspase-3 activation during normal development. The idea is that some cells die and need to be replaced so the cells that survive proliferate. Such an autonomous increase in proliferation might explain the abundance of GFP-expressing cells in the adult. It could also explain our otherwise paradoxical result that inhibition of executioner caspase activity in the wing imaginal disc by p35 reduced wing area in the adult. If inhibiting caspases only blocked apoptosis, one would expect the tissue to contain

excess cells and to be either larger, abnormally patterned, or both. In contrast we observed a small decrease in wing area, consistent with the idea that inhibiting caspase activity might also inhibit compensatory cell proliferation during normal development. An earlier study (de la Cova, Abril, Bellosta, Gallant, & Johnston, 2004) showed that inhibiting apoptosis in the wing disc led to variability in the size of the disc later in development; however this study did not address the ultimate effect on the size of the adult wing. It will be interesting in the future to examine CasExpress in models of injury, repair and regeneration to determine if cell autonomous compensatory proliferation occurs in those settings as well.

Additional examples of developmental anastasis

Two papers have documented examples of cell recovery from apoptosis during C. elegans development (Hoeppner, Hengartner, & Schnabel, 2001; Reddien, Cameron, & Horvitz, 2001). When phagocytosis

was impaired, a fraction of cells that normally died were able to reverse the morphological signs of apoptosis, which are caused by caspase-3 activity. These cells not only survived, they differentiated. One interpretation of these findings is that phagocytosis normally occurs so early in the death process that it prevents anastasis. However development in C. elegans is far more stereotyped than it is in most organisms. In C. elegans the fate of every single cell is precisely determined. However in organisms with greater numbers of cells, cell survival or death is not thought to be a predetermined cell fate; rather there is a selection process in which cells compete (de Beco, Ziosi, & Johnston, 2012; Merino et al., 2015; Moreno & Rhiner, 2014; Vincent, Fletcher, & Baena-Lopez, 2013). Our results indicate that many cells in adult flies derive from cells that survive caspase activity at some point during their development. An alternative interpretation of the C. elegans studies is that the ability to survive and recover even after caspase-3

activation is a fundamental and ancient cellular property that evolved early and still exists in a latent form, even in an animal that does not normally need it. Even in C. elegans, the precise moment of engulfment is not predetermined; and it is not always the same cell that consumes the dying cell. Therefore in organisms with larger numbers of cells whose fates are far less predictable, it is unlikely that engulfment always occurs at a precise time point during the apoptotic process.

In summary, the results presented here demonstrate that it is not rare for cells to survive caspase-3 activation during normal Drosophila development, and such cells make a major contribution to normal adult tissues.

Using CasExpress, we revealed the detailed temporal and spatial patterns of cells that survive caspase-3 activity in vivo during development of Drosophila. Based on the observation of two distinct patterns, we proposed two different types of survival: one pattern is

suggestive of non-apoptotic caspase activity, while the other suggests the concept of developmental anastasis. To further test these ideas, we realized that the difference between non-apoptotic caspase activation and developmental anastasis is that anastasis by definition requires induction of apoptosis whereas non-apoptotic caspase activation, by definition, does not. Other members of the lab have used mutations in pro-apoptotic genes and found in fact that reduction of developmental apoptosis reduces CasExpress activity in those tissues we predicted were undergoing developmental anastasis without affecting those cell types we had deduced were experiencing non-apoptotic caspase activity. A key open question for the future is to uncover genes that are required for both types of CasExpress activity, especially in those tissues that the signals are likely to be developmental anastasis. This goal could be achieved by generating knock-out clones of genes or knocking down genes with shRNA. This is technically challenging because CasExpress uses UAS-Gal4

and FRT-FLP, the two systems that are most widely used to generate clones and knockdown gene expression. Thus current efforts in the lab include using parallel systems like QF/QUAS must be used as alternatives. An additional key open question is whether anastasis is critical for normal development, wound healing, or tumor. Demonstrating the molecular mechanisms and physiological significance of anastasis will be important future directions.

Materials and methods

Fly strains

Tub-Gal80ts (Bloomington #7018) is from Bloomington Stock Center.

Wing size quantification

Crosses were maintained at 25°C. The progeny of the desired genotypes were collected and dehydrated in 100% ethanol. The wings were then mounted in Canada balsam (Gary's magic mountant, Sigma) and photographed using a Zeiss AxioZoom microscope. Wing sizes were quantified using ImageJ software.

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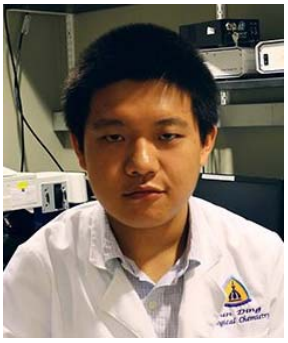

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